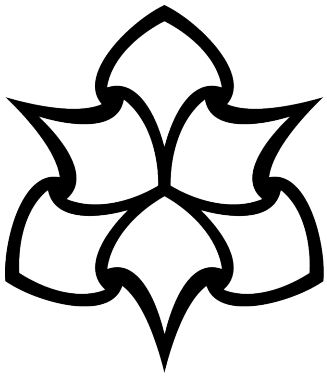

The Therapeutic Potential of Aged Garlic Extract in the Protection against Doxorubicin-Induced Cardiotoxicity

Huda Mohammed Alkreathy

A thesis submitted in partial fulfillment of the requirement of the Manchester Metropolitan University for the degree of Doctor of Philosophy

School of Healthcare Science
Manchester Metropolitan University

2011

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Acknowledgements

In the name of Allah who taught man about matters that he does not know and prayers and peace be upon our Holy prophet Mohammed the last of all God's prophets and his good followers till the day of judgement. Initially, I thank Allah who helped and guided me throughout my life.

I would like to thank my supervisors Drs Nessar Ahmed, Mark Slevin, Zoheir Damanhouri and Prof Abdel-Moneim Osman for their continuous support, encouragement and guidance throughout this study.

Professor Amaar Amine, the Director of the Joint Supervision Program, at King AbdulAziz University was always there to listen, solve problems and give support. I would like to express my deep and sincere gratitude to him for his understanding and encouragement in completing this thesis.

I am deeply grateful to Dr Alessandra Basouti for giving me training in RT^2qPCR technique and other molecular biology techniques. I am thankful to Dr. Ide Nagatoshi from Wakunaga of Americal Company (Wakunaga Pharmaceutical Company, Japan) for providing the aged garlic extract used in this study.

Thanks to all the technical staff of MMU especially, Mike Head and Bernard Ridings. I also thank my friend Khadijah Balamash for her encouragement, valuable comments and advice. I am graeful to Dr Soad Shaker for help in interpretation of histopathological study. I would like to convey special thanks to Prof Mansour Suliaman for his encouragement and care, and last but not least thanks to all friends who supported me. All the studies have been supported by Joint Supervision Program at King AbdulAziz University.

Finally, I strongly believe that, I could never have started and completed this work without the full, unconditional and endless support, guidance, assistance and patience of my husband and without the endless and full support of my parents.

Declaration

I declare that this work has not been accepted for any degree before and is not currently being submitted in candidature for any degree other than the degree of Doctor of Philosophy of the Manchester Metropolitan University

Huda Mohammed Alkreathy

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Alkreathy, H., Damanhouri, Z. A., Ahmed, N., Slevin, M., Ali, S. S. & Osman, A.-M. M. (2010) Aged garlic extract protects against doxorubicin-induced cardiotoxicity in rats. *Food and Chemical Toxicology,* 48**,** 951-956.

Alkreathy, H., Damanhouri, Z. A., Ahmed, N., Slevin, M. & Osman, A.-M. M. (2011) Mechanisms of cardioprotective effect of aged garlic extract against doxorubicin-induced cardiotoxicity. Integrative Cancer Therapies (accepted).

List of Abbreviations

BSA	Bovine serum albumin
DAPI	4',6-diamidino-2-phenylindole
dNTP	deoxynucleotide triphosphate
DADS	Diallyl disulphide
DAS	Diallyl sulphid
DATS	Diallyl trisulphide
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetra acetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Foetal bovine serum
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
H_2SO_4	Sulphuric acid
M	Molar
MgCL2	Magnesium chloride
Mg	Milligram

mL	Millilitre
MDA	Malondialdehyde
mRNA	Messenger ribonucleic acid
MTS	Tetrazolium and phenazine methosulfate (MTS reagent)
MAPK	mitogen-activated protein kinase
NaCl	Sodium chloride
NAD	β-nicotinamide adenine dinucleotide
NADH	Reduced nicotinamide adenine dinucleotide
NADPH	Reduced nicotinamide adenine dinucleotide phosphate
$NaHCO_3$	Sodium bicarbonate
Na/K-ATPase	Sodium-potassium pump
NaOH	Sodium hydroxide
NMR	Nuclear magnetic resonance
NO	Nitric oxide
$NO^{\cdot}$	Nitric oxide radical
$O_2^{\cdot}$	Superoxide radical
$OH^{\cdot}$	Hydroxyl radical
PBS	Phosphate buffer saline

PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT^2 qPCR	Rreal-time reverse transcription quantitative polymerase chain reaction
RT	Reverse transcription
PKC	Protein kinase C
PSG	Pencillin, Streptomycin and L-glutamine
$ROO^{\cdot}$	Peroxyl radical
ROS	Reactive oxygen species
SAC	S-allyl cysteine
SAMC	S-allyl mercaptocysteine
SEM	Standard error of mean
SOD	Superoxide dismutase
SPSS	Statistical Package for the Social Sciences 15.0 for Windows
TAS	Total antioxidant capacity
TCA	Trichloroacetic acid
cDNA	Complementary deoxyribonucleic acid
Ct	Cycle threshold
H&E	Haematoxylin and eosin

HRP	Horseradish peroxidase
WHO	World health organization
µg	Microgram
µL	Microlitre
µM	Micromolar

Abstract

Doxorubicin (DOX) is a one of the most potent anticancer drug which is widely used in the treatment of childhood and adult cancer. Cardiac toxicity is a dangerous so far unsolved complication of DOX. Doxorubicin-induced cardiotoxicity is attributed to oxidative stress and p53-dependent apoptosis. The establishment of an effective safe compound would be of great benefit in the management of DOX-induced cardiotoxicity. Aged garlic extract (AGE) is a natural, promising compound to lessen DOX-induced cardiotoxicity due to it antioxidant, antiapoptotic, and multiple health promoting effects. This study investigated the protective effect of AGE against DOX-induced cardiotoxicity in Wistar rats and in rat cardiac myocytes. It also investigated the effect of AGE pre-treatment on oxidative stress, p53, active caspase-3 and gene expression in DOX-treated rat cardiac myocytes. The results of this study have revealed that AGE protects against DOX-induced cardiotoxicity *in vivo* and *in vitro*. Four groups of rats were assessed for serum cardiac enzymes, plasma and heart malonialdehyde (MDA), serum total antioxidant status (TAS), and light and electron microscopic examination of the heart tissue. Serum cardiac enzymes were found to be elevated in DOX-treated rats. The findings of this study have revealed that there is an oxidative stress in DOX-treated rats, as manifested by increased plasma and heart MDA concentrations and reduced serum TAS. Pre-treatment with AGE reduced MDA concentrations and normalised TAS, which are indicators of oxidative stress in DOX-treated rats and attenuated histopathological alterations in DOX-treated rats. Aged garlic extract pre-treatment did not interfere with the cytotoxic activity of DOX, but it augmented DOX uptake into tumour cells in mice bearing EAC and increased the long term survivors of tumour-bearing mice. Pre-treatment of rat cardiac myocytes with AGE lowered DOX-induced elevation of 8-isoprostane, p53 and caspase-3 activity. The results of this study demonstrated that pre-treatment with AGE insignificantly reduced increased expression of some antioxidant

genes in DOX-treated rat cardiac myocytes. Further studies are needed to identify the detailed molecular mechanisms underlying the protective effects of AGE.

Chapter 1 General Introduction

1.1 Introduction to cancer

Cancer is a disease resulting from a change in the control mechanism that handles cell survival, proliferation, and differentiation. Any malignant growth or tumour caused by abnormal and uncontrolled cell division is known as cancer. Cancer may spread to other parts of the body through the lymphatic system or the blood stream. Cancer is one of the most common causes of death in the world. In 2010, cancer caused the deaths of close to 1,500 Americans each day. Notably, it is the second most common cause of death in the US, exceeded only by heart disease. Furthermore, cancer is responsible for nearly 1 in every 4 deaths. In addition, it should be highlighted that cancer is not a single disease; rather, it is a group of diseases characterised by the uncontrolled growth and spread of abnormal cells.

Moreover, age is a known risk factor for the development of cancer. Essentially, cancer is very much a disease of aging with the exception of a few cancers, such as childhood leukaemia and testicular cancer. In older adults, there is a rapid increase in the incidence of cancers, such as, breast, melanoma, prostate and ovarian (Ferlay *et al.*, 2010). Various factors account for the increased incidence of cancer in the elderly, including hormonal changes, accumulated damage (e.g., excessive sun exposure, smoking), and age-related declines in the immune function (Beghe and Balducci, 2005) and decreased efficiency in cellular mechanisms (e.g., changes in telomerase activity). Moreover, numerous studies have demonstrated an association between immunosuppressive treatment and the development of tumours (Vajdic *et al.*, 2006).

Incidence of cancer

Cancer is a major cause of death worldwide: during 2007, it was responsible for 7.9 million deaths (approximately 13% of all deaths). The majority of cancer-related deaths each year are owing to cancer of the lungs, stomach, liver, colon and breast.

There is a difference between men and women in the most common types of cancer. Breast cancer is the world's most common female cancer, with approximately 1.4 million cases occurring annually, thereby accounting for almost one quarter of all cancers in women (Ferlay *et al.*, 2010). Furthermore, twelve million deaths worldwide as a result of cancer are estimated to occur in 2030.

According to the IARC GLOBOCAN 2008 database, an estimated 12.7 million new cancer cases and 7.6 million deaths were been reported as of 2008. The most commonly diagnosed cancers worldwide include lung (1.61 million; 12.7% of the total), breast (1.38 million; 10.9% of the total) and colorectal cancers (1.23 million; 9.7% of the total). Furthermore, the most common causes of cancer-related deaths are owing to cancer of the lung (1.38 million; 18.2% of the total), stomach (0.74 million; 9.7% of the total) and liver cancers (0.69 million; 9.2% of the total) (Ferlay *et al.*, 2010).

Risk of developing cancer

The majority of cases of cancer have been reported in adults who are middle-aged or older. Moreover, approximately 78% of all cancers are diagnosed in persons aged 55 years and older. The probability that an individual will develop cancer during the course of their lifetime or die from cancer is described as lifetime risk. According to the American Cancer Society Statistics 2010, men have slightly less than a 1 in 2 lifetime risk of developing

cancer, whilst women have a risk of a little more than 1 in 3. The most important lifestyle risk factor for cancer is tobacco use (Lopez *et al.,* 1994; McCormack and Boffetta, 2011).

Cancer in Saudi Arabia

The national cancer registry in Saudi Arabia was established in 1992; however, there have been annual incidence reports ranging from 1992 through to 2005. The number of cancer cases and the incidence of cancer are both increasing. From the ninth incidence report published by the Saudi Cancer Registry in 2005, the overall age-standardised incidence rate (ASR) for all Saudis with a world standard population reference was 74.3/100,000 (74.1/100,000 in males and 74.4/100,000 in females). For all sites, the age-specific incidence rate (AIR) increased with age for both males and females. After the age of 64 years, the increase was almost double for males compared with females. The median age at diagnosis was 59 years for men and 49 years for women.

The five geographic regions with the highest ASR were the Eastern region at 98.2/100,000, Riyadh region at 91.2/100,000, Makkah region at 80.5/100,000, Tabuk region at 70.6/100,000, and Jouf region at 61.9/100,000. The total number of cancer cases analysed amongst Saudis during the year 2005 was 7,563. Accordingly, Table 1.1 shows the most common cancers in Saudi Arabia, with lung cancer seen to account for approximately 4% of all newly diagnosed cancers in Saudi Arabia (Alamoudi, 2009).

Table 1.1: Ten most common cancers in the Saudi population in 2005 (all ages)

Cancer	Number	%
Breast	948	12.5
Colo-rectal	778	10.3
Non-Hodgkin lymphoma	596	7.9
Thyroid	473	6.3
Leukemia	441	5.8
Lung	347	4.6
Liver	292	3.9
Skin	281	3.7
Prostate	261	3.5
Hodgkin disease	259	3.4

Taken from (Alamoudi, 2009)

Treatment of cancer

Cancer treatment options include chemotherapy, radiation treatments, and surgery. Antineoplastic agents help cancer patients with varying degrees of success, and can be used either in combined regimen or separately.

Surgical treatment

For many centuries, surgery was the only treatment for cancer, and still remains the mainstream treatment for many forms of cancer despite the introduction of additional methods of therapy. Surgery is used to diagnose or treat cancer. When cancer has not spread to the lymph node or distant sites (metastasized), surgery is considered to be the most effective in treating and eliminating most types of cancer. Furthermore, surgery may be used alone or in combination with other treatments, including radiation therapy and chemotherapy. Moreover, surgery is curative if the cancer has not metastasised.

The treatment of cancer depends on the type, location and size of the tumour. In order for radiation therapy and chemotherapy to be more effective, surgery is applied in order to reduce tumour size. This procedure is known as debulking. Moreover, surgery has no significant role in widespread metastasised or inoperable tumours affecting the head and neck cancer.

Radiation therapy

The second most common form of treatment of cancer, next to surgical excision, is radiotherapy. It is estimated that 50% of cancer patients will be treated with radiotherapy at some time during the course of their disease. Radiation therapy has a central role in the management of many types of cancer either as the only treatment or as part of other treatment options, including surgery, chemotherapy or immunotherapy (Glatstein *et al.*, 2008).

The use of several types of ionising radiation (x-ray, gamma rays or electron beams) to treat tumours is a part of conventional cancer radiation therapy. Radiation therapy acts by causing damage to the DNA of tumour cells; this results in biochemical alterations, which subsequently lead to the arrest of the cells' ability to divide indefinitely. Measured doses of radiation should be accurately related to an established tumour volume. Moreover, owing to the fact that radiation oncologists aim to balance the desired damage to the tumour and the undesirable radiation-induced injury to adjacent tissues, lethal tumour doses are not always accomplished (Levin *et al.*, 2005).

In the case of tumours adjacent to critical body structures, appropriate targeting and delivery of radiation dose is essential. Charged-particle-beam therapy which has been clinically available since 1954 is an important technology which is able to achieve precise delivery of radiation dose.

Chemotherapy for the treatment of cancer

The majority of patients with cancer present to their doctor when the disease has already spread. For these patients, the surgical resection of the primary tumour or local radiotherapy

may control the tumour, but cure of the metastatic disease via such treatments is impossible. Thus, for the majority of cancer patients, the cure of the disease depends either upon earlier diagnosis tumours being treated when local treatment may be curative, or otherwise upon the development of some systemic treatment to kill metastatic tumour cells.

The origin of cancer chemotherapy can be traced back to the 1860s, during which time potassium arsenate was used in the treatment of leukaemia (Forkner and Scott, 1931; Waxman and Anderson, 2001).

The early results of cancer chemotherapy were not impressive, but in 1941, oestrogens were shown to cause regression of metastics prostate cancer; this was the first real evidence that cancer cells could be controlled via drug treatment (Haddow, 1943; Cox and Crawford, 1995). During the last twenty-five years, cancer chemotherapy has evolved to become a part of a sound medical discipline with an important role in the control of cancer. There are quantitative rather than qualitative biochemical differences between normal and neoplastic tissues; nevertheless, such quantitative differences can be utilised as a basis for chemotherapy.

In order to overcome the limited log kill of individual anticancer drugs, a combination of agents with varying toxicities and mechanisms of action are used. This combination chemotherapy is the standard approach to the curative treatment of testicular cancer and lymphomas, and also to palliative treatment of many other tumour types.

Chemotherapy destroys not only the cancer cells but also many rapidly dividing normal body cells, such as the cells lining the gastrointestinal tract, hair follicles, bone marrow cells and lymphocytes (Hu *et al.,* 2011; Kris *et al.,* 2011). This normal cell destruction leads to the

common side effects experienced with chemotherapy, including nausea, vomiting, diarrhoea, hair loss and greater susceptibility to infection.

Types of chemotherapeutic agents

There are many different classifications for anticancer chemotherapeutic agents; they can be classified according to their source into synthetic compounds and natural products. Moreover, those originating from plant or bacterial sources are known as natural. Approximately one quarter of anticancer drugs in use are derived from natural products (Bernardes *et al.*, 2009; Demain and Vaishnav, 2010; Karikas, 2010). Furthermore, synthetic agents of clinical use are alkylating and antimetabolite agents.

Another group is the anthracyclines, plant alkaloids, topoisomerase inhibitors (Takimoto, 2008). All of these drugs affect cell division, and DNA synthesis or function. Monoclonal antibodies and the new tyrosine kinase inhibitors, e.g. imatinib mesylate, act by directly targeting a molecular abnormality in certain types of cancer (George, 2002). Furthermore, hormones are used in the treatment of cancer; they modulate tumour cell behaviour without directly attacking cancer cells (Gundersen *et al.*, 1994).

1.2 Anthracycline antibiotics

History and mechanism of action

Anthracyclines rank amongst the most effective anticancer drugs ever developed (Weiss, 1992). The first anthracyclines were isolated from the pigment-producing *Streptomyces peucetius* early in the 1960s, and were named doxorubicin (DOX) and daunorubicin (DNR) (Di Marco et al., 1969). Several other anthracycline analogs are in use in clinical practice, including idarubicin, epirubicin, and mitoxantrone. Moreover, anthracyclines (ACs) are used

in the treatment of haematologic and solid malignancies owing to the fact that they are a highly effective chemotherapeutic agent (Hortobagyi, 1997).

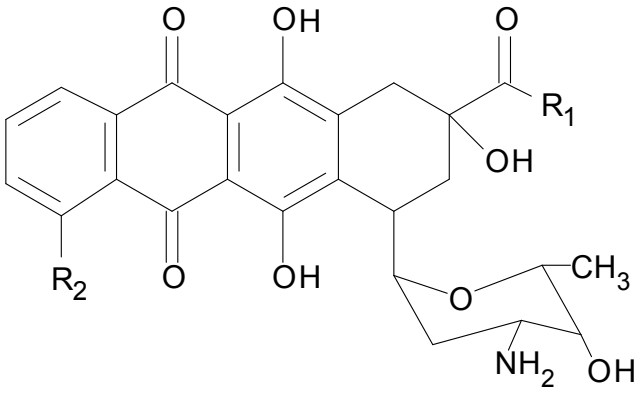
They have weak bases and have a planar cyclic anthraquinone nucleus attached to an amino sugar (Figure 1.1). Moreover, they have the ability to interfere with rapidly diving cells. This action is mediated through their ability to intercalate into cell DNA (Gewirtz, 1999). The major antitumour effect of this drug involves DNA intercalation and interference with the catalytic cycle of DNA topoisomerase II (Hande, 1998; Lyu *et al.*, 2007). The planar aglycone moiety (Figure 1.1) can insert non-specifically between adjacent base pairs of DNA, and binds to the sugar phosphate backbone of DNA from the minor groove (Hande, 1998; Zeman *et al.*, 1998; Palchaudhuri and Hergenrother, 2007), thereby causing local uncoiling of DNA strands, thus resulting in a block of DNA and RNA synthesis, as well as the inhibition of DNA repair. Intercalation can also interfere with the topoisomerase II-catalyzed breakage reunion reaction of DNA strands to cause a non-repairable break.

Chemistry of doxorubicin

Doxorubicin has a tetracycline chromophore ring attached by glycosidic linkage with daunosamine sugar (Figure 1.1). It also has a quinine and hydroquinone moieties on adjacent rings which modify its function as an electron-accepting and donating agent. The carbohydrate moiety is essential for its antitumour activity (Henry, 1979), and has a molecular weight of 579.98 and is soluble in water at 50mg/ml. Doxorubicin hydrochloride is stable with no loss of activity when reconstituted with sterile water and refrigerated at 4^{o}C for six months (Hoffman *et al.*, 1979).

Pharmacokinetics of doxorubicin

Doxorubicin is very poorly absorbed following oral administration, and is therefore commonly administered through intravenous infusion in saline over a period up to 96 hours in order to reduce cardiotoxicity (Myers and Chabner, 1990). It is administered commonly in a dose of 60 to 75 mg/m^2 as a single intravenous injection at 21-day intervals when used as a single agent (Benjamin *et al.*, 1974). In addition, DOX is used also simultaneously with other chemotherapeutic drugs. The usual dose is 40 to 60 mg/m^2 as single intravenous injection every 21–28 days; this is repeated for 4–6 or more cycles.



	R_1	R_2
Dororubucin:	CH_2OH	OCH_3
Danorubicin:	CH_3	OCH_3
Idarubicin:	CH_2OH	H
Epirubicin:	like doxorubicin with epimerization of the hydroxyl group on the aminosugar	

Figure 1.1: Chemical structure of anthracyclines

Doxorubicin follows a multiphasic disposition after intravenous injection. The disappearance curve of DOX is biphasic with elimination half-lives of 0.6 h and 16 h (Rodvold *et al.*, 1988; Cusack *et al.*, 1993). There is a rapid tissue uptake of DOX, as is suggested by the initial distribution half-life of approximately 5 minutes. As the drug is distributed into tissue, DOX blood levels fall dramatically. The terminal half-life of 20–48 hours results in slow elimination from tissues (Creasey *et al.*, 1976; Benjamin *et al.*, 1977). Moreover, there is an extensive drug uptake into tissues, as is indicated from the steady state distribution volume of 809 to 1214 L/m^2. The drug does not cross the blood brain barrier; rather, the slow elimination phase of DOX is owing to renal and biliary clearance and metabolism.

Doxorubicin is metabolised in the liver. Doxorubicin metabolism occurs through the reduction of a side chain carbonyl group by aldoketo reductases (Ahmed *et al.*, 1981), subsequently resulting in the formation of the metabolite doxorubicinol. Moreover, it is also metabolised by a reductive cleavage of the sugar moiety to form the 7-hydroxy aglycone (Pan and Bachur, 1980). Doxorubicin and its major metabolite doxorubicinol binding to plasma protein is 74–76%. Furthermore, 40% of the dose appears in the bile in 5 days, whilst 5–12% of the drug and its metabolite appear in the urine during the same time period.

There is a correlation between DOX partitioning from blood to tissue and DNA concentration (Terasaki *et al.*, 1982; Terasaki *et al.*, 1984). Recently, analytical analyses of DOX reactions using nuclear magnetic resonance (NMR) spectroscopy have highlighted that DOX can form covalent adducts with DNA at guanine-cytosine (GC) sequences, with a bond formed between DOX aminosugar N3’ and guanine N2’ of the DNA strand (Wang *et al.*, 1991; Cutts and Phillips, 1995; Swift *et al.*, 2008; Cipolla *et al.*, 2009). The formation of DOX covalent adducts with DNA has been correlated to programmed cell death (Zeman *et al*., 1998); however, further studies have shown additional antitumour effects of DOX,

including binding of negatively charged phospholipids in cell membranes with resultant alterations of their function, metal ion chelation, and generation of free radicals.

Many studies have also shown that DOX favourably binds to the cardiolipin of mitochondrial membranes, thereby resulting in defects of mitochondrial membrane functions (Marcillat *et al.*, 1989; Goormaghtigh *et al.*, 1990). The maximum antitumour activities of DOX occur during S-phase of the cell cycle. At low concentrations, cells will proceed through S-phase and then die in G2-phase.

Side effects of doxorubicin

Nausea, vomiting, diarrhoea and loss of appetite are some of the common side effects. Doxorubicin also causes temporary alopecia, stomatitis. A major dose-limiting complication is myelosuppression (Bally *et al.*, 1990; Judson *et al.*, 2001). Another toxic manifestation as a result of DOX treatment is hepatotoxicity (Bagchi *et al.*, 1995). Cardiotoxicity is the most serious complication and it limits the total dose.

A dose-dependent drug-induced cardiotoxicity which can progress to irreversible congestive heart failure has limited the widespread use of anthracyclines (Figure 1.2). The overall prevalence of DOX cardiomyopathy is 1.7–6.8% (Cortes *et al.*, 1975; Lefrak *et al.*, 1975; Minow *et al.*, 1975; Praga *et al.*, 1979), and is highly dependent on the total dose. Moreover, there is a sharp increase in the incidence of DOX-related cardiotoxicity at accumulative dose above 550 mg/m^2 body surface area. Furthermore, it is recommended that the maximum cumulative dose of DOX is 500 or 450 mg/m^2.

A large-scale study that retrospectively evaluated the cardiotoxicity of DOX reported that an estimated 7% of patients developed DOX-related congestive heart failure (CHF) following a cumulative dose of 550 mg/m^2 (Swain *et al*., 2003). Heart damage following anthracycline

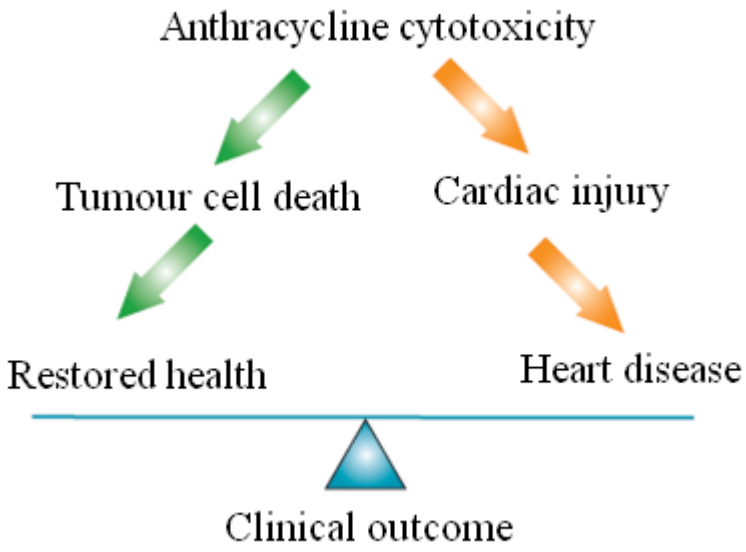


Figure 1.2: The cytotoxicity of anthracycline antibiotics. (Adapted from Peng *et al.*, 2005)

chemotherapy can be divided into early and late cardiotoxicity. Cardiotoxicity that develops during chemotherapy or otherwise during the first year following its completion is considered to be an early cardiotoxicity, whilst if this occurs one year or more following the completion of therapy, it is defined as a late cardiotoxicity (Shan *et al*., 1996). Patients can show DOX-cardiotoxicity as subclinical heart failure and as clinical heart failure. The incidence and risk of early clinical cardiotoxicity and late subclinical cardiotoxicity have been evaluated by several studies (Sallan and Clavell, 1984; Sorensen *et al.*, 1995; Nysom *et al.*, 1998).

Risk factors for doxorubicin cardiotoxicity

There is an increased risk for both types of cardiotoxicity alongside a higher cumulative dose of DOX (Minotti *et al*., 2004) with the female gender, age over 65 years (but also very young children), diabetes, pre-existing heart disease and hypertension, liver disease or mediastinal radiotherapy, type of tumour, black race, presence of trisomy 21, and exposure to cyclophosphamide, ifosfamide, or amsacrine (Dearth *et al.*, 1984; Lipshultz *et al.*, 1995; Puma *et al.*, 2008; Velensek *et al.*, 2008).

Mechanism of action of doxorubicin

The mechanisms of cytotoxicity of DOX in cancer cells include four main mechanisms: firstly, the inhibition of topoisomerase II; secondly, high affinity binding to DNA, via nucleic intercalation, which leads to the inhibition of DNA and RNA synthesis, and cleavage of DNA strands by alterations of topoisomerase II; thirdly, binding to the cell membrane, which changes its normal fluidity and transport of ions; and finally, the generation of semiquinone free radicals and oxygen free radicals through an iron-dependent, enzyme-mediated reductive process (Davies and Doroshow, 1986; Gervasi *et al.*, 1986; Minotti *et al.*,

2001). Tumour cell growth is inhibited in response to some or all of these effects, and cells are more likely to die by one or more of these mechanisms (Gewirtz, 1999). Furthermore, it occurs in normal tissues as well as the tumour target, and the effects on the heart pose a major clinical dilemma (Figure 1.2). Doxorubicin causes cardiotoxicity by mechanisms other than those mediating their antitumour effectiveness, and so strategies can be developed which protect the heart without diminishing tumour response.

1.3 Mechanism of doxorubicin cardiotoxicity

In spite of the wide use of DOX, the cardiotoxic mechanism is still not completely understood. The most common hypothesis is the formation of free radicals and superoxide (Rajagopalan *et al*., 1988; Vasquez-Vivar *et al*., 1997). Cardiac cells are more prone to free radical damage owing to their highly oxidative metabolism and relatively poor antioxidant defences (Doroshow *et al*., 1980). This mechanism was proposed originally as the basis for DOX related cardiotoxicity, although other variables may be involved, including the interaction of the DOX with iron (Myers *et al.*, 1977). Doxorubicin can interact with haem-containing cellular proteins, such as complex I of the mitochondrial respiratory system, so as to generate damaging reactive oxygen species (Figure 1.3).

Accordingly, mitochondria play a major role in the action of DOX particularly with respect to cardiotoxicity (Jung and Reszka, 2001; Conklin, 2005). Abnormal mitochondria are one of the earliest and most prominent histomorphological features of acute anthracycline-induced cardiomyopathy. Several studies demonstrate that DOX affects both ATP and phosphocreatine levels in cultured cardiac cells (Seraydarian *et al.*, 1977). Furthermore, defects of several bioenergetic functions in the mitochondria have been reported (Iwamoto *et*

al., 1974; Mailer and Petering, 1976; Goormaghtigh *et al.*, 1982; Muhammed *et al.*, 1982; Muhammed *et al.*, 1983; Muhammed and Kurup, 1984; Praet *et al.*, 1984).

It has been shown that DOX forms an electrostatic complex with cardiolipin a phosholipid of the mitochondrial inner membrane (Goormaghtigh *et al.*, 1982; Goormaghtigh *et al.*, 1983; Praet *et al.*, 1984; Goormaghtigh *et al.*, 1986). Cardiolipin is required by mitochondrial complex I,II,III and IV in order to maintain maximal activity (Fry and Green, 1981). Moreover, DOX can undergo a one-electron reduction, which can result in the formation of a semiquinone radical species. Furthermore, mitochondrial complex I catalyses DOX reduction (Thayer, 1977; Davies *et al.*, 1983; Davies and Doroshow, 1986; Doroshow and Davies, 1986). One electron transfer to molecular oxygen leads to the re-oxidation of the semiquinone radical species, which accordingly leads to the formation of superoxide anion. In addition, activated oxygen species such as superoxide, hydrogen peroxide and hydroxyl radicals will be formed from the interaction of DOX products with NADH dehydrogenase of Complex 1 (Davies *et al.*, 1983; Davies and Doroshow, 1986; Doroshow and Davies, 1986).

It is also known that DOX can activate signal transduction pathways and cause apoptosis in cardiac myocytes, as has been shown in previous studies (Wu *et al.*, 2002; Clementi *et al.*, 2003; Mihara *et al.*, 2003; Rebbaa *et al.*, 2003). Moreover, DNA fragmentation and DNA degradation occur as result of DOX-induced apoptosis. Furthermore, there is also simultaneous activation of many apoptotic-associated protein activities and levels, including caspase-3 (Rebbaa *et al.*, 2003), caspase-9 (Cui *et al.*, 2002), cytochrome C (Clementi *et al.*, 2003), and reduced levels of anti-apoptotic proteins, such as Bcl-2 (Wu *et al.*, 2002). Doxorubicin causes the activation of pro-apoptotic proteins and increased levels of BAX (Wu *et al.*, 2002) and p53 proteins (Cui *et al.*, 2002)

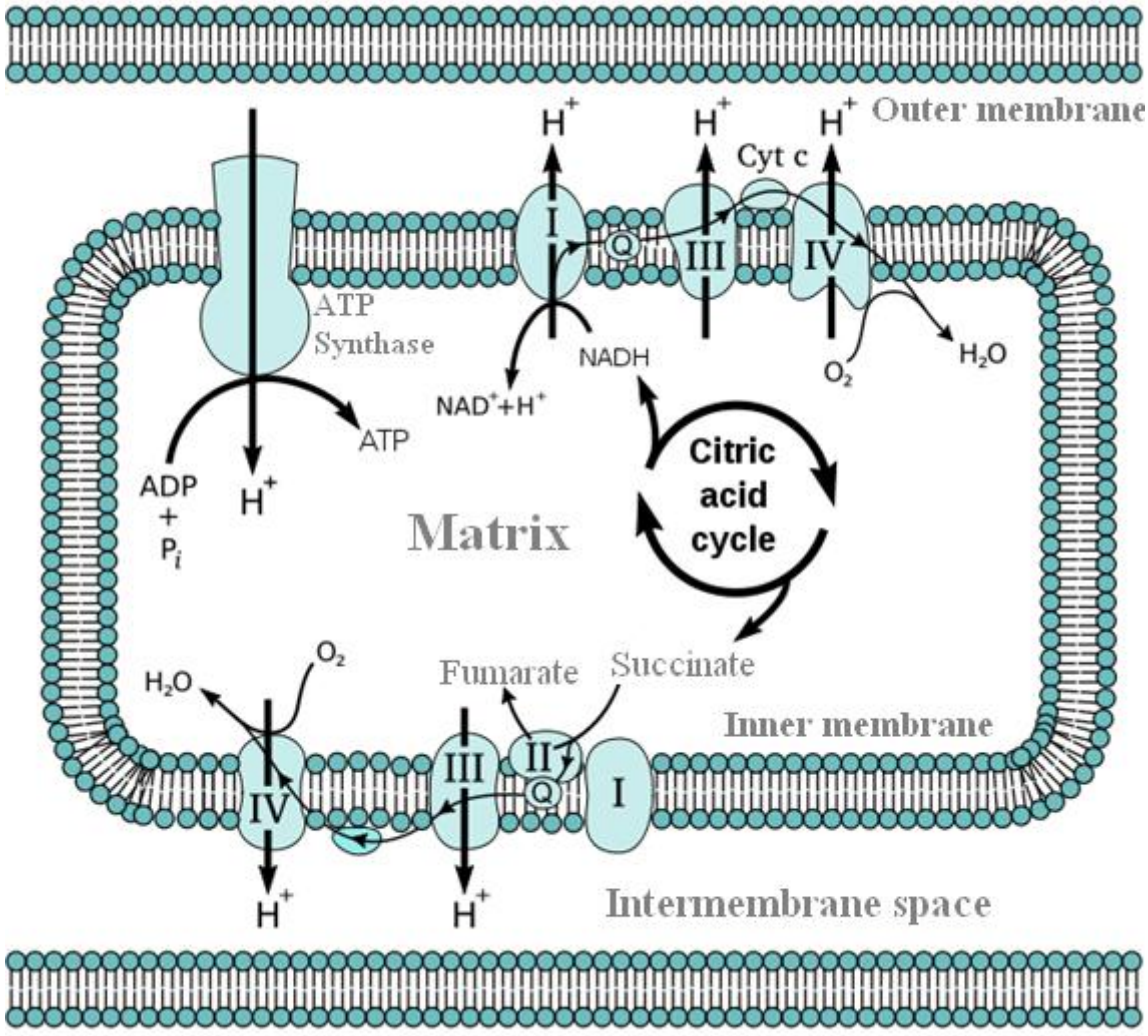


Figure 1.3: The electron transport chain in the mitochondrion.

Doxorubicin cardiotoxicity is described as Type 1 chemotherapy related cardiac dysfunction (Ewer and Lippman, 2005), which is characterised by ultra-structural changes, and has a greater susceptibility to become irreversible. Myofibrillar loss, dilation of sarcoplasmic reticulum and swollen mitochondria are the major morphological changes in the myocardium of patients treated with DOX. Furthermore, mitochondrial respiration provides more than 90% of ATP that is utilised by cardiomyocytes; therefore, any change of mitochondrial structure and function will result in disturbance in cardiomyocyte function (Ventura-Clapier *et al.*, 2004).

1.4 Oxidative stress and doxorubicin cardiotoxicity

Oxidative stress has been implicated in the pathology of different diseases, including cancer, diabetes mellitus, inflammatory disease, ageing and also in DOX-induced cardiotoxicity (Figure 1.3). Moreover, normal cellular metabolism produces reactive oxygen species (ROS). Reactive oxygen species at low concentrations result in beneficial effects, and have a physiological role in the host defence mechanism (against infectious agents) and in a number of cellular signalling systems (Barja, 1993). Notably, the overproduction of reactive oxygen and/or a deficiency of antioxidant mechanisms results in a harmful state, which is known as oxidative stress (Halliwell, 1994). Notably, excess ROS inhibit normal functions in the body as it damages cellular DNA, lipids, and protein.

Owing to the effect of unstable free radicals on various cellular molecules, such as lipids, DNA and proteins, human beings are subjected to various diseases and aging. The reactive oxygen species damage often leads to disrupting enzymes, ultimately developing mutations causing cancer, injuring membranes, and decreasing overall immunity. It has also been established that ROS are the resultant of normal metabolism. Furthermore, it can be

neutralised through small molecules and cellular antioxidant enzymes (Queiroza *et al*., 2009). Some of these small molecules include minerals, vitamins and phytochemicals.

The level of ROS is often found to increase during inflammation, or otherwise when a person is exposed to sunlight for a long. Other causes of increase in ROS levels include exercise, radiation, pollution, smoking and even some medicines. The increase in ROS is often seen to lead to oxidative stress, which further results in heart diseases, arthritis, aging, HIV/AIDS, cancer, atherosclerosis, stroke, Alzheimer's disease (Amagase *et al.*, 2001; Borek, 2001; Banerjee *et al.*, 2003; Dillon *et al.*, 2003).

Molecules containing one or more unpaired electrons are known as free radicals (Gilbert, 2000). Free radicals have a high degree of reactivity owing to the unpaired electrons (Bergendi *et al.*, 1999). As a consequence of their instability, free radicals tend to either accept or donate electrons from or to other adjacent molecules in order to achieve a more stable state, thereby leading to a propagation of chain of reactions with the formation of new radicals, which in turn can react with further macromolecules. Lipid peroxidation is an example of free radical-mediated tissue damage (Betteridge, 2000). The most common class of radical species are those derived from oxygen. With this in mind, all aerobic organisms use molecular oxygen to generate ATP, which is the chemical energy considered useful for life. The end products of many catabolic pathways are combined with O_2 in the mitochondria to produce most of this energy.

In mammalian cells, mitochondrial ATP-production takes place mainly in enzyme complexes coupled with the electron transport chain (McIntyre *et al.*, 1999). During energy transduction, a small number of electrons leak out, subsequently forming the free radical superoxide. Superoxide is produced from both complexes I and III of the electron transport

chain (Miwa *et al.*, 2003) and, in its anionic form, can readily cross the inner mitochondrial membrane. Excessive production of superoxide under stress conditions releases free iron from iron-containing molecules, such as haemoglobin. This released iron can generate hydroxyl radical (through the Fenton reaction) (Liochev, 1999; Thomas *et al.*, 2009). The hydroxyl radical, •OH, has a very short half-life (approximately 10–9 s) and indiscriminately oxidises its closest targets (Kehrer, 2000).

Other sources of ROS include cellular enzyme systems, such as NADPH oxidase, xanthine oxidase, uncoupled endothelial nitric oxide (NO) synthase (eNOS), arachidonic acid metabolising enzymes comprising cytochrome P-450 enzymes, lipoxygenase and cyclooxygenase (Griendling, 2005; Mueller *et al.*, 2005). Besides, free radicals may be generated in the body in response to electromagnetic radiation from the environment, and acquired directly as oxidising pollutants, such as ozone and nitrogen dioxide (Betteridge, 2000; Irmak *et al.*, 2002). Furthermore, increased levels of transition metal ions such as those of iron, copper and mercury, which are potent catalysts for free radicals formation lead to the generation of free radicals via the Fenton chemistry (Valko, 2005; Valko *et al.*, 2006).

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + \bullet OH + OH^-$$

In order to protect the cells and organ systems of the body against reactive oxygen species, humans have developed a highly complex antioxidant protection system that normally scavenge free radicals produced by the Fenton reaction and other stimuli; thus, antioxidants are capable of stabilising or deactivating free radicals before they attack cells. Hydroxyl, peroxyl ($\bullet RO_2$), alkoxyl (RO•), and hydroperoxyl ($HRO_2^{\bullet}$) are examples of oxygen-free radicals. Examples of nitrogen-free radicals include Nitric oxide (•NO), and nitrogen dioxide ($NO_2^{\bullet}$). Oxygen- and nitrogen-free radicals can be converted to other non-radical reactive

species, including hydrogen peroxide, hypochlorous acid (HOCl), hypobromous acid (HOBr), and peroxynitrite ($ONOO^-$) (Figure 1.4) (Fang *et al.*, 2002).

An antioxidant is defined as any substance that when present at low concentrations compared with those of an oxidizable substrate, significantly delays or completely prevents oxidation of that substrate (Halliwell and Gutteridge, 1995; Gutteridge and Halliwell, 2000). Enzymes are the first line of protection against ROS. Superoxide dismutase (SOD), catalase, glutathione reductase, glutathione peroxidase and glutathione S-transferase are all examples of antioxidant enzymes (Rhrdanz and Kahl, 1998; van Deel *et al.*, 2008). There are also non-enzymatic antioxidising agents which act as a second line of defence against ROS. Nonenzymatic antioxidants include glutathione, ascorbate (vitamin C), α-Tocopherol (vitamin E), carotenoid, flavonoid and α-lipoic acid (Ryan *et al*., 2010; Krishnaiah *et al.*, 2010).

Cardiotoxicity of DOX is known to be partly mediated through the generation of ROS. The findings highlighted that the treatment of animals with a variety of antioxidants such as probucol, amifostine, dexrazoxane, and melatonin protects heart against the toxicity of DOX support the role of ROS in DOX-induced cardiac toxicity (Seifert *et al.*, 1994; Siveski-Iliskovic *et al.*, 1994; Samelis *et al.*, 1998; Nazeyrollas *et al.*, 1999; Liu *et al.*, 2002). In addition, DOX-induced cardiac injury is greatly attenuated when antioxidant enzymes, such as manganese superoxide dismutase (MnSOD), catalase, or glutathione peroxidase 1 (Gpx1), in the cardiomyocytes of transgenic mice are overexpressed (Kang *et al.*, 1996; Yen *et al.*, 1996; Xiong *et al.*, 2006).

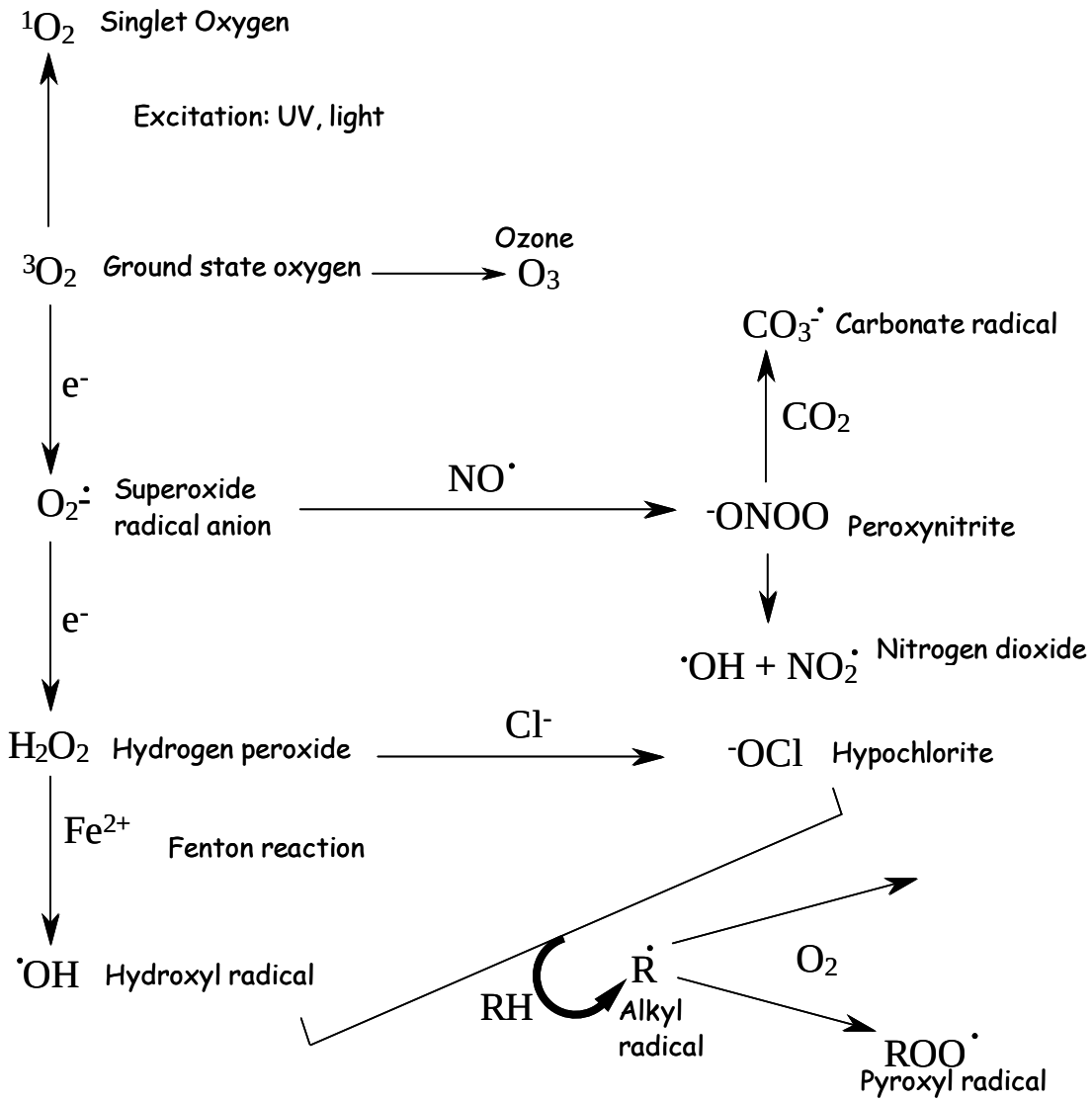


Figure 1.4: Main reactive oxygen species; RH: organic molecule. (Adapted from Bartosz, 2009)

The previous studies strongly support the role of ROS in DOX-induced cardiotoxicity because MnSOD specifically transforms superoxide to hydrogen peroxide. Mitochondria is the major target of DOX-induced toxicity in cardiomyocytes as MnSOD is only located in the mitochondria (Goormaghtigh *et al.*, 1983; Davies and Doroshow, 1986; Keller *et al.*, 1998; Ashley and Poulton, 2009).

1.5 Strategies to reduce doxorubicin cardiotoxicity

Use of analogues

During the last two decades, there have been numerous attempts to identify a novel anthracyclines superior to DOX in terms of activity, which produces less cardiac toxicity. This resulted in the synthesis of 2000 analogues (Weiss, 1992). Amongst these analogues, only a few have been approved clinically. Epirubicin (EPI) and idarubicin (IDA) are popular useful alternatives to DOX or DNR, respectively. Unfortunately, replacing DOX with EPI or IDA does not abolish the risk of developing chronic cardiotoxicity (Anderlini *et al.*, 1995; Ryberg *et al.*, 1998).

Alternative approaches to drug delivery

Administration of DOX as continuous slow infusion over 48–96 hours instead of the standard rapid infusion has been associated with less cardiac toxic effects (Legha *et al.*, 1982; Shapira *et al.*, 1990). Furthermore, the use of liposomes to target drug delivery to reduce cardiotoxicity has been tested in clinical trials, and was found to be effective (Treat *et al.*, 1990; Strother and Matei, 2009).

Combination therapy with antioxidants

The use of agents that may counteract the free-radical mediated cardiotoxic effect of DOX without interfering with its antitumour effect could be of value. Injac and Strukelj (2008) recently reviewed the protective effects associated with several natural products, drugs, adjuvant therapy, and numerous approaches, including exercise and calorie restriction.

The primary mechanism of DOX -induced cardiotoxicity is the production of free radicals as a by-product of DOX metabolism. This suggests some new approaches, such as the potential use of natural antioxidants (Quiles *et al.*, 2002). Vitamins (E, C, A, carotenoids), coenzyme Q, flavonoids, polyphenols, selenium, herbal antioxidants, and virgin oil are the most commonly used and investigated compounds (Quiles *et al.*, 2002).

Probucol is a lipid-lowering drug which has been found to be effective in protecting the heart against the toxic effects of DOX (Li and Singal, 2000). Probucol is a strong antioxidant owing to the presence of two phenolic groups in its molecular structure. Li and Singal (2000) investigated the effect of probucol on DOX-induced cardiotoxicity in a rat model, and accordingly reported that probucol completely hindered cardiotoxicity without interfering with the antitumour effect of DOX. Moreover, Probucol prevented myocardial lipid peroxidation and DOX-induced decrease in antioxidant activity (Iliskovic and Singal, 1997). Glutathione peroxidise (GSHPx) activity is also increased in rat heart by probucol.

Another drug which has been shown as equally effective as probucol is the pineal hormone melatonin. Morishima *et al.* (1998) studies rats, and subsequently demonstrated that melatonin may be protective against DOX-induced cardiotoxicity through the inhibition of lipid peroxidation in rats.

Combination therapy with iron chelator

Moreover, several studies have investigated the role of iron chelator in the protection against DOX cardiotoxicity. Herman and Ferrans (1981), for example, revealed that dexrazoxane reduced the abnormalities associated with DOX in dog heart. Another study by (Osman *et al.*, 1993) demonstrated that desferrioxamine attenuated the haematological and cardiotoxic complication of DOX in mice. The cardioprotective effect of dexrazoxane is owing to its ability to inhibit the conversion of Fe^{3+} to Fe^{2+}, thereby leading to the inhibition of formation of hydroxyl radical. Moreover, Schroed and Hasinoff (2002) demonstrate that the open ring hydrolysis product of dexrazoxane, ADR-925, is able to remove Fe^{3+} from its complex with DOX in rat. Dexrazoxane is clinically proven for reducing DOX-induced cardiotoxicity (Schroeder and Hasinoff, 2002). In addition, Lebrecht *et al.* (2007) reported recently that dexrazoxane prevents late-onset DOX cardiotoxicity by preventing cardiac mitochondria from interconnected genetic and functional insults, which are triggered off and maintained by ROS. Unfortunately, however, with dexrazoxane, there are increases in the incidence of leukopenia (Cvetkovic and Scott, 2005).

So far, there is no specific treatment for cancer therapy-related cardiomyopathy. Symptomatic patients receive standard treatments for congestive heart failure, such as angiotensin-converting enzyme inhibitors, beta-blockers, diuretic, digoxin and spironolactone (Simbre *et al*., 2001). However, recent experimental evidence supports the preventive effects of erythropoietin on cardiac dysfunction in DOX-induced cardiomyopathy (Li *et al.*, 2006).

1.6 Garlic

Garlic is one of the most investigated medicinal plants. Garlic (*Allium sativum*) has been known for centuries for its various roles as a medicine. The oldest reports of health-promoting properties of garlic date back to the 16th Century BC when, in the so-called Ebers Papyrus from Egypt, over 20 aliments were purported to be efficiently cured by garlic (Block, 1985). In the last 15 years, interest has arisen in attempting to identify the specific medicinal properties of garlic, as well as its active ingredients responsible for therapeutic effects.

Moreover, the raw garlic contains sulphur compounds such as alliin, ajoene, cysteine sulfoxides and trisulfides, and at least 17 amino acids in addition to a variety of vitamins and minerals (Brace, 2002) (Table 1.2). Water is a major constituent of aged garlic extract (AGE). It also contains other compounds, such as carbohydrate, sulphur compounds, protein, fibre, amino acids, saponins, vitamins and minerals (Lawson, 1996). Organosulphur compounds are responsible for the characteristic flavour and aroma associated with garlic. In AGE, most of the components responsible for the characteristics, such as odour (thiosulfinates), are removed during the aging process. Water-soluble organosulphur compounds together with unique biochemical constituents, such as S-allyl-L-cysteine, fructosylarganine and 1,2,3,4-tetrahydro-β-carboline-3-carboxylic acids are the major elements present in AGE (Ryu and Rosen, 2003). The conversion of γ-glutamyl-S-allylcysteine the parent compound to alliin occurs during the aging process to produce S-allylcysteine (SAC), S-allylmercaptocysteine (SAMC) and others. S-allylcysteine is used for standardization because it is bioavailable (Nagae *et al.*, 1994).

Besides its dietary consumption, the use of garlic supplementation for its therapeutic benefits is becoming more and more popular worldwide. It has been shown that garlic can act as an antithrombotic, antihypertensive, antiplatelet (Agarwal, 1996; Rahman, 2001), antimicrobial, antiatherosclerotic, antihypoglycemic, anticancer, antidote (for heavy metal poisoning), hepatoprotective and immunomodulatory agent (Agarwal, 1996; Banerjee *et al.*, 2003).

Commercial garlic products

Several garlic preparations exist in the market, including garlic tablets, aged garlic extract, oil of steam-distilled garlic, oil of oil-macerated garlic, ether extracted oil of garlic, and liquid garlic (Table 1.3).

Aged garlic extract

Wakunaga of America Co. Ltd. (Mission Viejo, USA) manufactures AGE. The soaking of sliced raw garlic in 15–20% aqueous ethanol for up to 20 months in stainless steel tanks at room temperature result in the formation of AGE. Aged garlic extract tablets, powder capsules and liquid forms are a result of the filtration and concentration of aged garlic in ethanol under reduced pressure at low temperature. Aged garlic extract liquid form contains 10% (w/v) ethanol. Thiosulphinates, the harsh and pungent oil soluble constituent in garlic is present in garlic preparations other than AGE. Furthermore, AGE is rich in mild and odourless water soluble sulphur compounds. Adverse effects, such as aggravation of the stomach, hepatotoxicity and oxidasing activity against red blood cells, are caused by thiosulphinate allicin (Egen-Schwind *et al.*, 1992a; Egen-Schwind *et al.*, 1992b; Burden *et al.*, 1994; Freeman and Kodera, 1995; Hoshino *et al.*, 2001). On the other hand, AGE has proven safe for human consumption, with is no toxicity associated with its prolonged use ((Hoshino *et al.*, 2001; Lawson and Gardner, 2005).

Table 1.2: Commercially available garlic products

Product	Main compounds and characteristics
Garlic essential oil	I% oil-soluble sulphur compounds (e. g., DAS or DADS) in 99 % vegetable oil
	No water-soluble fraction
	No allicin
	Not well standardised
	No safety data
Garlic oil macerate	Oil-soluble sulphur compounds and alliin
	No allicin
	Not well standardised
	No safety data
Garlic powder	Alliin and a small amount of oil-soluble
	sulphur compounds
	No allicin
	Not well standardised
	Results on cholesterol are not consistent
	No safety data
Aged garlic extract	Mainly water-soluble compounds (e. g.
	SAC, SAMC or saponins)
	Standardised with SAC
	Small amount of oil-soluble sulphur compounds
	Various beneficial effects
	Well-established safety
	Heavily researched (300+ papers)

Taken from Amagase et al. (2001)Abbreviations: DAS, diallyl sulphide; DADS, diallyl disulphide; SAC, s-allyl cysteine;SAMC, s-allyl mercaptocysteine.

Table 1.3: General composition of garlic

Component	Amount (fresh weight ;%)
Water	62-68
Carbohydrates	26-30
Protein	1.5-2.1
Amino acids:common	1-1.5
Amino acids: cysteine sulphoxides	0.6-1.9
y-Glutamylcysteine	0.5-1.6
Lipids	0.1-0.2
Fibre	1.5
Total sulphur compounds	1.1-3.5
Sulphur	0.23-0.37
Nitrogen	0.6-1.3
Minerals	0.7
Vitamins	0.0015
Saponins	0.04-0.11
Total oil-soluble compounds	0.15
Total water-soluble compounds	97

Taken from (Lawson 1996)

Chemical constituents of aged garlic extract

Aged garlic extrac provides similar health benefits as those achieved by fresh garlic, albeit without the presence of unlikable side effects of fresh garlic (Imai *et al.*, 1994; Nagae *et al.*, 1994; Amagase *et al.*, 2001; Borek, 2001; Kasuga *et al.*, 2001). Moreover, AGE is considered to be a very high-standard product being developed from organic fresh garlic that is aged and extracted for around 20 months at room temperature. During this process, the antioxidants found in garlic are increased to very high levels compared with those found in fresh garlic. Furthermore, the process helps to convert the harsh and not-so-stable compounds, such as allicin, and to produce a much more stable and palatable substance

Potential therapeutic effects of aged garlic extract

Over 360 scientific research studies have been published which attest to the health benefits of AGE and it constituents.

Boosting immunity

The immune system of human beings has various types of protective substances and cells which help to fight several infections, as well as life-threatening illnesses, including cancer. Moreover, a person with a strong immune system is also able to fight viral, bacterial and fungal diseases without much difficulty; however, in cases where the immune system suffers damage such as in the case of HIV/AIDS patients, or owing to aging, pollution, stress, malnourishment etc. The human body is not able to fight the infection, and often succumbs to the ailment. In this regard, studies have found that AGE helps to improve the immune system and to fight infections and diseases (Adetumbi and Lau, 1983; Kyo *et al.*, 2001; Nance *et al.*, 2006).

Antiviral properties

Research has indicated that AGE helps to prevent the influenza virus from spreading, and is found to be as effectual as vaccination. It has also been found that AGE aids in terms of enhancing immunity in patients suffering from HIV/AIDS; it also increases the natural killer cells (NK) activities which help to kill cancer cells and to stimulate activity in various immune cells, including macrophages and lymphocyte. Therefore, AGE assists in enhancing the anti-cancer activities of the immune cells (Deshpande *et al.*, 1993).

Abdullah *et al*. (1989) found that, following the inclusion of AGE in the diet of patients suffering from AIDS for 6 weeks, there were marked improvements witnessed in their NK cells activities, which were previously depleted owing to the AIDS virus. However, AGE treatment increased the NK cells to the normal levels again.

Antibacterial and antifungal properties

Clinical studies have found that, owing to the utilisation of AGE, the development of *Candida albicans* yeast can be inhibited. Yeast is often viewed as the main reason for oral infections in patients with HIV/AIDS, as well as other sexually transmitted diseases (Tadi *et al.*, 1990). Moreover, it has also been found that AGE helps to kill *Heliobacter pylori*, which is predominantly linked with cancer and stomach ulcers. In addition, further research may reveal that AGE might help to cure *Heliobacter pylori* as it has been found that approximately 85% of patients with *Heliobacter pylori* do not respond to antibiotics (Delaha and Garagusi, 1985)

Antiallergic properties

Owing to the rise in pollution and environmental disruption, allergies have become a part of human life. In this regard, the majority of allergies result from the release of mast cells by histamine, which can cause havoc in our lives. However, studies have also established that AGE may help to prevent the release of histamine by between 50% and 90%. Furthermore, it also helps to decrease allergic reactions by approximately 25–45%, even after a person is exposed to various allergy-inducing substances (Kyo *et al.*, 1997).

Anti-stress properties

In the traditional medicines, practitioners have often prescribed garlic to help eradicate stress and enhance vigour, which has been highlighted through various studies. Moreover, additional researches have also established that AGE has aided swimmers and runners to enhance their endurance (Ushijima *et al.*, 1997; Morihara *et al.*, 2006; Morihara *et al.*, 2007). Furthermore, as per a study in Japan, patients with stress showed improved results when they were given AGE supplements, along with Vitamins B12 and B1. Markedly, it was also found that AGE helped to reduce fatigue and weaknesses in the patients (Hasegawa *et al.*, 1983).

Preventing cancer

DNA mutation which has been aggregated over a period of time, combined with the risk factors associated with age, is known to result in the occurrence of cancer. It has been found that chemical carcinogens and injury by free radicals are the main causes of damage to DNA (Amagase and Milner, 1993). With this in mind, a study has found that the intake of garlic in

diet increases immunity and lowers the risks associated with the growth of colon, stomach and prostate cancer (Steinmetz *et al.*, 1994; Fleischauer and Arab, 2001).

Studies also indicate that AGE may help to prevent various kinds of cancer, as it scavenge free radicals which may potentially damage DNA, thereby enhancing antioxidant levels, blocking carcinogens and increasing the process to dispose carcinogens. Therefore, AGE may aid in reductions of cancer of the liver, bladder, lungs, mammary glands and oesophagus, along with colon, stomach and prostate (Milner, 1996).

With this in mind, a number of preclinical studies provide evidence that garlic may potentially inhibit carcinogen-induced tumours in various organs (Milner, 1996). It has been reported that there is a significant reduction in gastric cancer risk with increasing consumption of garlic in humans (You *et al.*, 1989).

It has also been found that AGE can also aid in enhancing cancer therapy. As per analysis, the major death-related cancers are breast, prostate and colon cancer; however, recent studies indicate that S-allyl mercaptocysteine and S-allyl cysteine found in AGE may help to inhibit the growth of cancer cells in the prostate by approximately 80%. Furthermore, it is also acknowledged that AGE also helps to prevent the growth of cancer cells in the prostate, thereby preventing polyamines used to divide cells and accordingly decreasing PSA or prostate-specific antigen (Pinto *et al.*, 1997, Pinto *et al*., 2000).

Minimising drug toxicity

During the treatment of cancer, the major problem or issue that one may face is liver toxicity and cardiotoxicity arising owing to ROS produced by drugs, such as methotrexate, DOX and fluorouracil. Research has found that AGE can help to protect the liver from fluorouracil and

methotrexate toxicity (Yüncü *et al.*, 2006); thus, AGE can be potentially used to cure patients who are on anti-cancer therapy. Furthermore, AGE even helps to reduce toxicity in the liver owing to environmental pollution and carcinogens (Wang *et al.*, 1998; Pinto and Rivlin, 2001). In addition, AGE is a neuroprotector as it was found to inhibit amyloid β-protein (Abeta) toxicity (Kosuge *et al.*, 2003).

In human beings, AGE has been seen to help to reduce risks factors associated with cardiovascular diseases (Imai *et al.*, 1994; Steiner *et al.*, 1996; Rahman and Billington, 2000 Borek, 2001; Ho *et al.*, 2001; Lau, 2001; Dillon *et al.*, 2002). Aged garlic extract decreases blood pressure and cholesterol, and also lowers homocysteine (Dillon *et al.*, 2002). Furthermore, AGE also improves blood circulation and decreases the progression of atherosclerosis by approximately 50%, thereby leading to reductions in heart attack risks (Srivastava *et al*., 1995).

Antioxidant effects of aged garlic extract

Research has found that AGE is loaded with antioxidants compared with the fresh garlic and various other garlic preparations available in the market. Aged garlic extract helps to augment cellular antioxidants and to aid maintaining a healthy body and immune system, along with preventing toxicity (Wei and Lau, 1998). Park *et al*. (2009) investigated the antioxidant activities and antigenotoxic effects of garlic extract prepared through various different processing methods, and concluded that garlic extract possesses significant protective effects against DNA damage induced by hydrogen peroxide (H_2O_2) and four-hydroxynonenal (HNE) that may be related to antioxidant activity.

Moreover, various studies have found that garlic has anti-oxidant properties which may help to increase the immune system and to also prevent strokes and heart diseases. In addition,

studies have also found that AGE helps to prevent the decrease of glutathione (GSH) where in oxidised LDL and endothelial cells are put together (Ide and Lau, 2001).

Ide and Lau. (1999) have demonstrated that in a cell free system, AGE scavenges H_2O_2. In addition, AGE can guard the endothelial cells from oxidized LDL-induced injury by preventing depletion of intracellular GSH and by removing peroxides. AGE boost GSH levels in vascular endothelial cells by regulation of the GSH redox cycle specifically increasing glutathione disulphide (GSSG) reductase activity. Furthermore, an increase in SOD activity was also observed (Zhaohui Geng, 1997).

Aged garlic extract possesses strong antioxidant activity. Drobiova *et al*. (2009) have reported that the treatment of streptozotocin-induced diabetic rats with 500 mg/kg garlic daily caused increased antioxidant activity reaching levels in excess of those observed in normal rats. Moreover, Kim *et al*. (2001) suggested that SAC act as a scavenger of superoxide radical and it also increases Cu/Zn SOD activity. It was shown that a dose of 125 mg/kg i.p. of SAC produced a decrease in superoxide radical production and blocked (100% of protection) of lipid peroxidation in mice (Rojas *et al*., 2011).

The antioxidant activity of AGE and SAC in hydroxyl radical and superoxide generating systems was measured by Kim *et al*. (2001). They found that the formation of 5,5-dimethyl-1-pyrrolline N-oxide (DMPO) adduct of the hydroxyl radical was strongly prevented by garlic extract and SAC in the H_2O_2 plus iron system which produces the hydroxyl radical. In addition, AGE and SAC reduced the accumulation of superoxide generated in the xanthine oxidase (XO)/acetaldehyde system.

Dillon *et al.* (2002) investigated the antioxidant properties of AGE *in vivo* in non-smoking and smoking human volunteers. The levels of 8-iso-$PGF_{2\alpha}$ are a reliable and sensitive novel

marker of increased oxidative stress and lipid peroxidation in vivo. It was used to evaluate the antioxidant effects of AGE. Dietary supplementation with AGE for 14 day significantly lowered plasma and urine concentrations of 8-iso-prostaglandin $F_{2\alpha}$ by 29% and 37% in non-smokers and by 35% and 48% in smokers.

Aged garlic extract and cardioprotection

Aged garlic extract has been shown to protect against cardiovascular disease (Imai *et al.*, 1994; Steiner *et al.*, 1996; Rahman and Billington, 2000; Borek, 2001; Ho *et al.*, 2001; Lau, 2001; Dillon *et al.*, 2002). Furthermore, AGE is also known to reduce blood pressure, lower LDL and elevate HDL cholesterol, inhibit the production of prostaglandins involved in inflammation and vasoconstriction, lower homocysteine, and also inhibit platelet aggregation and adhesion. Furthermore, it has been reported by Harauma and Moriguchi (2006) that AGE reduced blood pressure in spontaneously hypertensive rats more safely than raw garlic. Aged garlic extract protects also against dementia, which could be related to its effects in increasing microcirculation and lowering homocysteine (Dillon *et al.*, 2002; Yeh and Yeh, 2006).

The synthesis of constitutive nitric oxide a protective element against myocardial damage is increased by AGE (Morihara *et al.*, 2002). Studies demonstrate that AGE improves microcirculation and blood properties by preventing lipid peroxidation and haemolysis in oxidised erythrocytes (Moriguchi *et al.*, 2001).

Researchers state that AGE helps to lower blood pressure, high cholesterol, high homocysteine level and triglycerides, and also reduces risks associated with stroke and heart diseases. It has also been seen that AGE along with S-allyl cysteine aid in decreasing the risks associated with most of these diseases (Bordia, 1981; Fulder, 1989; Warshafsky *et al.*,

1993; Murray, 1995). Moreover, in their study, Steiner and Li (2001) found that an intake of 2.4–4.8 grams of AGE daily for six months helps to reduce cholesterol by around 5–7%. The study also found that AGE aided in lowering LDL, preventing the collection of platelets that might promote clotting, and decreasing blood pressure and triglycerides. It further assisted in increasing HDL, which promoted a healthy body.

Various studies have also found that garlic and its compounds help in inhibiting enzymes, which cause fatty acid synthesis and cholesterol in rats and human cells (Gebhardt, 1993; Liu and Yeh, 2001; Yeh and Liu, 2001). After measuring the enzyme activity, it was also found that garlic and its compounds prevent the synthesis of enzymes which cause cholesterol biosynthesis. These enzymes are namely HMG-CoA reductase and squalene monooxygenase

Moreover, it has been found that AGE helps to reduce cholesterol in the same manner as statin drugs, which are taken to lower cholesterol levels. Liu and Yeh (2002) studied the effects of water-soluble organosulphur compounds of garlic on hepatic cholesterol biosynthesis in cultured rat hepatocytes, and accordingly found that the activity of 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase in the cells treated with AGE was 30–40% lower than that of the untreated cells. Moreover, it has also been seen that AGE taken in combination with statins in patients with known coronary artery disease help in the suppression of cholesterol in a better manner. Moreover, AGE may exert anti-atherogenic effects through the inhibition of both smooth muscle phenotypic change and proliferation, and on lipid accumulation in the artery wall and to the macrophage (Budoff *et al.*, 2004). However, it has also been seen that AGE taken alone may also provide similar benefits of lowering cholesterol without the side effects, such as muscle pain and fatigue, as felt by some people who take statin regularly (Liu and Yeh, 2002; Budoff *et al.*, 2004).

Several other cardioprotective influences of garlic has also been found in a number of studies. For instance, garlic is being used to reduce unstable angina, to enhance the elasticity of blood vessels and to diminish arterial occlusive diseases (Breithaupt-Grogler *et al.*, 1997; Li *et al.*, 2000). The effect of AGE as a cardioprotective against DOX toxicities requires investigation at both *in-vivo* and *in-vitro* level.

Hypothesis

Administration of AGE will offer cardioprotection in rats given cardiotoxic doses of DOX.

1.7 Aims of the Study

The aims of this study were to:

- Investigate the protective effect of AGE against DOX–induced cardiotoxicity in rats;
- Investigate the effect of AGE on the antitumor activity of DOX in mice.
- Investigate the protective effect of AGE against DOX–induced cardiotoxicity in rat cardiac myocyte; and
- Investigate the effect of AGE on the signalling pathway and gene expression *in vitro*.

Chapter 2 : Materials and Methods

2.1 Materials

Rat cardiac myocytes 4 million, (LONZA,UK)

Ehrlich ascites carcinoma cells (EAC), a generous gift from Prof Abdel-Moneim Osman

Active caspase-3 (R & D Systems International, UK)

BIO-RAD protein estimation kit (Bio-Rad laboratories, Hertfordshire, UK).

Bovine serum albumin (Sigma, UK)

Dulbecco's modified eagle's medium (Cambrex Bioscience)

Foetal bovine serum (FBS) (Cambrex Bioscience)

Dimethylsulphoxide (DMSO) (Sigma, UK)

Isopropanol, ethanol, Tween 20 (Sigma, UK)

Isoton 11, azide-free balanced electrolyte solution (Coulter)

Paraformaldehyde (Sigma, UK)

Sodium bicarbonate (Sigma, UK)

Trypsin-10X, trypsin-EDTA solution (Sigma, UK)

Phosphate buffered saline (PBS): one tablet of PBS was dissolved in 100 ml d H_2O and autoclaved at 121°C for 15 min.

Rat cardiac myocyte basal medium (LONZA, UK)

Rat cardiac myocyte growth medium (LONZA,UK)

Lipid peroxidation assay kit (Calbiochem, USA)

Human/mouse active p53 assay kit (R&D Systems, UK)

Mouse total p53 assay kit (R&D Systems, UK)

8-Isoprostane EIA kit (Calbiochem, USA)

Human active caspase-3 quantikine ELISA kit (R&D Systems, UK)

Kyolic aged garlic extract was a generous gift from Wakunaga of America Co., Ltd.

Randox total antioxidant control cat no. NX2331 and total antioxidant status assay kit (Randox Laboratories, Antrim, UK)

CellTiter 96® AQueous One Solution Cell Proliferation Assay (Promega, UK)

Male Wistar albino rats (8– 10 weeks of age, 180–200 g body weight) from King Fahd Medical Research Center, King AbdulAziz University, Jeddah, Saudi Arabia.

Doxorubicin hydrochloride (Sigma, UK).

Plasma total lactate dehydrogenase (LDH) assay kit (Randox, UK)

Total creatine phosphokinase (CPK) assay kit (Spinreact, Spain)

Aprotinin, leupeptin, pepstatin , phenylmethylsulfonylfluoride (PMSF), chymostatin, sodium orthovanadate (Na3VO4) , activated sodium fluoride (NaF) , Sodium Azide (NaN3) , β-Glycerophosphate, Triton X-100 (Sigma, UK)

RT2 First Strand Kit, (SA Bioscience, USA)

Qiagen RNeasy® Mini Kit ,

AmpliTaq Gold® PCR Master Mix, 250 Units/ 5mL (Applied Biosystem, Roche, USA)

Oxidative Stress and Antioxidant Defense PCR Array (SA Biosciences, USA)

RT2 qPCR Primer Assay for rat Prdx5, RT2 qPCR Primer Assay for rat Ptgs2, RT2 qPCR Primer Assay for Rat Cygb, RT2 qPCR Primer Assay for rat Ucp3, RT2 qPCR Primer Assay for rat Gpx2, RT2 qPCR Primer Assay for rat Gpx7, RT2 qPCR Primer Assay for rat Ldha, (SA Bioscience, USA)

RT² qPCR SYBR Green/ROX MasterMix, (SA Bioscience, USA)

Tris-borate-EDTA (TBE) buffer,(Invitrogen,UK)

Ethidium bromide (Sigma, UK)

Vectashield mounting medium for fluorescence with DAPI (Vector Laboratories,USA)

Six-well, 24-well, 48-well and 96-well plates, 96-well EIA/RIA plates (Nunc Corporation)

Tissue culture flasks; T-25, and T-75 (Nunc Corporation)

Disposable tubes and stoppers, compatible with chloroform and methanol

Plain and EDTA tubes for blood collection. (BD Vacutainer®, USA)

Reusable glass 96-well plate. (Cayman Chemical, USA)

Centrifuge tubes 50 and 15 ml (Corning, USA)

2.2 Equipment

CO_2 incubator (Lab Impex Research)

Spectrophotometer (CECIL CE 7200, Cecil Instruments, Cambridge, UK)

Statistical package (version 15; SPSS Inc., Chicago) for the analysis of all the study data

Centrifuge (Sigma 3-16K, UK)

Single threshold coulter counter (Beckman Coulter) Water bath (LAUDA Brinkmann, Ecoline RE 120,Germany)

Kinetic microplate reader (Molecular Devices, USA)

LS 45 fluorescence spectrometer (Perkin-Elmer, USA)

Barnson Sonifier (250 VWR Scientific, Danbury, Conn, USA)

Digital multi channel pipits (Eppendorf)

Ice maker (Borolab Ltd)

Immunofluorescence microscope (Zeiss Axo imager Z1)

Inverted phase contrast microscope, Nikon TMS-F

Laminar flow hood or safety cabinet: tissue culture grade (Walker Safety Cabinet Ltd)

Magnetic stirrer hotplate (Stuart Scientific Co,UK).

Embitek RunOne-electrophoresis cell

Stratagene MX3000P

Universal model 200 laboratory, pH meter (Medical Scientific Instruments,UK).

Vortex (Chiltern, UK)

PerkinElmer Fluorescence Spectrometer LS55

Shaker (POS-300, Grant-bio, England)

Analytical scale (Sartorius, Oxford,UK)

PTC-100 Programmable-thermal cycle controller

PTC-200 Peltier thermal cycler, DNA engine

Ultrospec 2000, Phamacia Biotech, Cambridge, England.

Syngene G Box (Bio-imaging system) (Syngene, UK)

Biotek ELX800 Plate reader, USA.

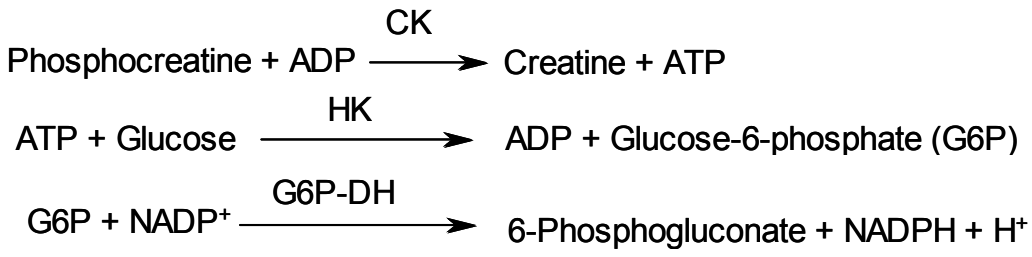
Deep freezer -80 ^{o}C ,Nuaire, Japan.

2.3 Measurement of serum total creatine phosphokinase

Creatine phosphokinase is a cellular enzyme with wide tissue distribution in the body. Its physiological role is associated with adenosine triphosphate (ATP) generation for contractile or transport systems. Elevated CPK values are caused by diseases of skeletal muscle and myocardial infarction (Antman *et al*., 2000). Serum CK activity raises following myocardial infarction beginning within 6 hours and peaking on an average at 24 hours and returning to normal within 2-3 days.

Principle

Serum total creatine phosphokinase (CPK) was measured using commercial kits from SPINREACT, Spain. Serum creatine phosphokinase was determined based on the method of Szazs *et al*. (1976). Creatine kinase (CK) catalysed the reversible transfer of a phosphate group from phosphocreatine to ADP. This reaction is linked to those catalysed by hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6P-DH):



The catalytic concentration of CPK present in the sample is proportional to the rate of NADPH formation, measured photometrically at 340nm.

Procedure

A volume of 20 µl aliquot of rat serum was pipetted into a cuvette, and then 1ml of working reagent was added and mixed well. Following incubation at 37^{o}C for 2 minutes, the initial absorbance (A) was read at 340nm. Thereafter, the absorbance (A) was recorded at 1 minute interval for 3 minutes. The difference between absorbances and the average absorbance differences per minute (ΔA/min) was calculated. The total CPK of the sample was calculated as A/minX8095 U/L CPK.

2.4 Measurement of serum lactate dehydrogenase

An increase in serum LDH activity is observed following myocardial infarction beginning within 6 – 12 hours and reaching a maximum at about 48 hours and it remains elevated for 4-14 days before coming down to normal levels. The prolonged elevation makes it a good marker for those patients admitted to the hospital after several days of myocardial infarction (Varley *et al*., 1984). Serum CPK and LDH activity were used as a marker for DOX cardiotoxicity in several studies (Al-Shabanah *et al.*, 1998, Yagmurca *et al*., 2003, Mansour *et al*., 2008, Koti *et al*., 2009, Ibrahim *et al*., 2010)

Principle

Serum lactate dehydrogenase (LDH) was assayed utilising the method of Moss and Henderson (1999). Lactate dehydrogenase catalysed the reduction of pyruvate by NADH, according the following reaction:

$$\text{Pyruvate} + \text{NADH} + \text{H}^{+} \xrightarrow{\text{LDH}} \text{L-lactate} + \text{NAD}^{+}$$

The rate of decrease in concentration of NADPH, measured photometrically at 340nm, is proportional to the catalytic concentration of LDH present in the sample.

Procedure

A volume of 50 µl aliquot of rat serum was pipetted into a cuvette, and then 1ml of working reagent was added and mixed well. Following incubation at 37^{o}C for 1 minute, the initial absorbance (A) was recorded at 340nm. The absorbance (A) was read at 1 minute intervals thereafter for 3 minutes. The difference between absorbances and the average absorbance differences per minute (ΔA/min) was calculated. The equation ΔA/min x 9690 = U/L LDH was used to calculate LDH.

2.5 Histopathological examination

The rat abdomen was opened under anesthesia and heart sample was removed immediately washed with saline. Part was processed for light microscope and other part for electron microscopic examination.

Structure

Heart pieces (approximately ½ cm) were fixed in 10% formaldehyde for 48 hours, and accordingly processed for paraffin sectioning at 5 µm thickness. The sections were mounted on glass slides and stained with haematoxylin and eosin to study the changes in the normal structure of the heart (Monnet and Orton, 1999).

Ultrastructure

For transmission electron microscopy, small pieces of heart tissue (1mm) were excised and fixed in 2.5% buffered glutaraldehyde overnight, and then post-fixed in 2% osmium

tetroxide for two hours at 4^{o}C. The specimens were then washed twice in phosphate buffer, dehydrated in ascending grades of ethanol, cleared in three changes of propylene oxide, and finally embedded in Epon 812 at 60^{o}C for two days. Sectioning was carried out on an LKB ultramicrotome with the use of glass knives for both semithin and ultrathin sections. The semithin sections (0.5 µm thickness) were stained using toluidene blue whilst the ultrathin sections (400 A thickness) were double stained with uranyl acetate and lead citrate, and examined under a Philips 300 electron microscope operating at 80 k,v (Robinson *et al*., 1987).

2.6 Measurement of plasma malondialdehyde

Kit components

Reagent 1: N-methyl-2-phenylindole in acetonitrile

Reagent 2: Methanesulfonic acid (MSA).

MDA Standard: 1,1,3,3-tetramethoxypropane (TMOP) in Tris-HCl.

Ferric ion in methanol: diluent for reagent 1.

Principle

Lipid peroxidation is well known to result from oxidative stress. Peroxidation of polyunsaturated fatty acids results in the formation of malondialdehyde (MDA) as an end-product. The measurement of MDA in the plasma provides an appropriate index of lipid peroxidation. Moreover, a colorimetric assay using the calbiochem assay kit was applied to measure MDA. The principle of this assay relies on the ability of the chromogenic reagent (R1) to react with MDA at 45^{o}C. Notably, a stable chromophore results from the

condensation of one molecule of MDA with two molecules of the chromogen reagent. This stable product has maximal absorbance at 586nm (Liu *et al*., 1997).

Standard preparation

A 10 mmol MDA solution was diluted 1/500 (v/v) in distilled water immediately prior to use to provide 20 µmol stock standard solution. Six standards solutions, each 200 µl were prepared for calibration by diluting the MDA standard (20 µM) with water to give the following concentrations: 0, 0.5, 1.00, 2.00, 3.00, and 4.00 µmol. Each standard was run in triplicate.

Procedure

Plasma samples were separated by centrifugation at 3000 g at 4^{o}C for 10 minutes. A volume of 650 ul of diluted reagent 1 was then added to each 200 ul sample in polypropylene tube and vortexed for 3–4 seconds. Subsequently, 150 µl of 12 N HCl was added, mixed well, and incubated at 45^{o}C for 60 minutes. Following, the tubes were cooled on ice, and the absorbance measured at 586nm. Next, a blank containing water was used to zero the spectrophotometer.

Calculation of MDA Concentration

From the MDA standard curve data, the net absorbance for each standard at 586nm was calculated by subtracting the blank ($A_{)}$ value from each of the standard absorbance values. Net (A 586) was plotted versus [MDA], and a linear regression analysis of A586 on [MDA] was performed as follows:

A_{586}=a [MDA] + b

The calculation of the MDA concentration in each sample was then calculated from the net A_{586} of the sample according to the following:

$$MDA = \frac{A_{586} - b \times df}{a}$$

where

[MDA] is the M concentration of MDA in the plasma

A_{586}= net absorbance at 586nm of the sample

A=regression coefficient (slope)

b=intercept

df=dilution factor

2.7 Measurement of serum total antioxidant status

Principle

Total antioxidant status was determined according to the method of Miller *et al.* (1993). This method is based on the quenching of 2, 2'-Azino-di(3-ethyl benzthiazolin sulphonate) radical cation ($ABTS^{\bullet+}$) by antioxidants; this was carried out with the use of a total antioxidant assay kit. In this assay, ABTS was incubated with a peroxidase, and H_2O_2 to produce the radical cation $ABTS^{\bullet+}$, resulting in a stable blue green colour measurable at 600nm. Antioxidants in the added sample cause inhibition of colour development to a degree proportional to their concentration.

Procedure

One ml of Chromagen was added to 20 µl aliquot of rat serum in a cuvette and mixed well. Following incubation at 37°C, the absorbance (A1) was recorded, and then 200 µl of H_2O_2 was added and mixed well. Three minutes later, the absorbance (A2) was recorded. The absorbance of a blank sample (20 µl deionized water) and a standard sample (20 µl) was assessed in the same way as serum samples. Calculation of the total antioxidant status of the sample was carried out using the formula:

$$\text{Factor} = \frac{\text{Conc. of standard}}{(\Delta \text{ A blank} - \Delta \text{ A standard})}$$

Total antioxidants (mM) = Factor × (Δ A blank - Δ A standard). The absorbance of total antioxidants (TA) in the serum is linear up to 2.5 mM.

2.8 Measurement of 8-isoprostane in rat cardiac myocyte culture medium

The isoprostanes are described as a part of eicosanoids which are non-enzymatic in nature. These are produced when tissue phospholipids are randomly oxidised through the use of oxygen radicals. As per a recent study, 8-isoprostane has been found to be the best index for determining oxidative injury through the use of an oxidant stress rat model (Gross *et al.*, 2005; Morrow, 2005). The level of 8-isoprostane in rat cardiac myocyte culture medium cells was determine using Cayman 8-isoprostane assay kit.

Kit Component

8-isoprostane EIA antiserum, 8-isoprostane AChE tracer, 8-isoprostane standard, EIA buffer concentrate (10X), wash buffer concentrate (400X), Tween 20, mouse antirabbit IgG coated plate, Ellman´s reagent.

Principle

Competition between 8-isoprostane and an 8-isoprostane acetylcholinesterase (AChE) conjugate (8-isoprostane tracer) for a limited number of 8-isoprostane specific rabbit antiserum binding sites forms the basis of this assay. The amount of 8-isoprostane tracer that is able to bind to the rabbit antiserum is inversely proportional to the concentration of 8-isoprostane in the well; this is owing to the fact that the concentration of 8-isoprostane tracer is held constant whilst the concentration of 8-isoprostane varies. The rabbit antiserum 8-isoprostane (either free or tracer) complex binds to the rabbit IgG mouse monoclonal antibody, which has been previously attached to the well. Subseqently, the plate is washed to remove any unbound reagents, and next, the Ellmans reagent containing the substrate to AChE is added to the well. This enzymatic reaction results in the formation of a product with distinct yellow colour, which was measured at 412nm. The strength of this colour is equivalent to the amount of 8-isoprostane tracer bound to the well, which is inversely proportional to the amount of free 8-isoprostane in the well during incubation.

Absorbance α [bound 8-isoprostane tracer] α 1/[8-isoprostane]

Procedure

A volume of 50 µl of EIA buffer and 50 µl of culture medium was added to non-specific binding wells. Then 50 µl of culture medium was added to maximum binding (B_o) wells. A volume of 50 µl from each of the eight standards which were diluted in culture medium (0.8-500 pg/ml) was added to standard wells in duplicate. The remaining wells were coated with 50 µl/well of culture medium sample. A volume of 50 µl of 8-isoprostane tracer was added to each well with the exception of the total activity (TA) and the blank wells. Next, 8-isoprostane EIA antiserum (50 µl) was added to each well with the exceptions of the total activity well, the non-specific binding, and the blank wells. Subsequently, the plate was covered with a plastic film and incubated for 18 hours at 4^{o}C. Later, the wells were emptied and rinsed five times with wash buffer. A volume of 200 µl of Ellman's reagent, which was reconstituted immediately before use, was added to each well. Next, 5 µl of tracer was added to the total activity wells. Next, the plate was covered with plastic film and placed on an orbital shaker in dark for 90–120 minutes at room temperature in order to allow the plate to develop. Finally, the plate was read at a wavelength 405nm.

Calculation of 8-isoprostane concentration

The mean of non-specific binding well absorbance readings was calculated. The mean of maximum binding (B_o) wells absorbance readings was calculated. The mean of the non-specific binding was subtracted from mean of B_o; this is referred to as the corrected B_o or corrected maximum binding. Next, the percentage sample or standard/maximum bound (%B/ B_o) was calculated for the remaining wells; this was done by subtracting the mean non-specific binding absorbance from the standard or sample absorbance, and the result was then divided by the corrected B_o. The detection limit of this assay is (80% B/ B_o), 2.7 pg/ml.

2.9 Measurement of active caspase 3 in rat cardiac myocyte cells

Kit component

Active Caspase-3 Microplate 96 well microplate (12 strips of 8 wells) coated with monoclonal antibody specific for caspase-3.

Active Caspase-3 Conjugate Concentrate 0.75ml of a 23-fold concentrated solution containing streptavidin conjugated to horseradish peroxidase (HRP), with preservatives.

Type 12 Conjugate Diluent: 12.5ml of buffer for diluting the conjugate concentrate, with preservatives.

Active Caspase-3 Standard: 40 ng of caspase-3 derivatised with a biotinylated inhibitor in a buffered protein base, with preservatives, lyophilised.

Calibrator Diluent RD5-20 Concentrate (5X): 21ml of a concentrated buffered protein solution, with preservatives.

Extraction Buffer Concentrate (5X): 21ml of a concentrated buffered protein solution containing surfactants, with preservatives.

Biotin-ZVKD-fmk Inhibitor: 400 g of biotinylated ZVKD-fmk inhibitor, lyophilised.

Wash buffer concentrate- 21ml of a 25-fold concentrated solution of a buffered surfactant, with preservative.

Colour Reagent A: 12.5ml of stabilised hydrogen peroxide.

Colour Reagent B: 12.5ml of stabilised chromogen (tetramethylbenzidine).

Stop Solution: 23ml of diluted hydrochloric acid

Principle

Active caspase-3 concentrations were measured by a quantitative sandwich enzyme immunoassay technique. The active Caspase-3 ELISA uses a biotinylated caspase inhibitor to covalently alter the large subunit of caspase-3. The inhibitor is added directly to the culture medium where it enters apoptotic cells and makes a stable thio-ether bond with the cysteine on the active site. Notably, the inhibitor does not covalently alter inactive caspase-3, which is the basis for discrimination between active and inactive caspase-3. Subsequently, cells are solubilised in a denaturing extraction buffer and diluted in order to decrease denaturant concentration. Caspase-3 specificity is obtained through the use of a caspase-3 specific monoclonal antibody coated on the microplate. Both caspase-3 zymogen and the large subunit are captured by the monoclonal antibody. Detection is with HRP-streptavidin which binds the biotin on the inhibitor attached to the caspase-3 large subunit. Owing to the fact that the zymogen is not modified with biotinylated inhibitor, it is not detected by HRP-streptavidin. The ELISA measures the relative amount of caspase-3 large subunit modified with biotin-ZVKD-fmk (fluoromethylketone). Owing to the fact that the modification requires that the large subunit is present in an active caspase-3, the amount of active caspase-3 is directly proportional to the amount of biotin-ZVKD-fmk-modified large subunit

Procedure

Following the induction of apoptosis in cardiac myocyte, 2 µl of 5 mM biotin-ZVKD-fmk per 1ml of culture medium was added to obtain a final concentration of 10 µM. Cells were incubated with biotin-ZVKD-fmk inhibitor for 1 hour. Following the 1 hour incubation in the CO_2 incubator, the medium was removed and cells were gently rinsed with phosphate

buffered saline (PBS). A volume of 110 µl extraction buffer (1X) containing protease inhibitors was added. Next, cells were scraped with plastic 200 µl pipette tips. Following, the six well plates was covered and placed in refrigerator at 2–8^{o}C overnight. The subsequent day, 400 µl per well of calibrator diluents was added and the 6 well plate was rocked to mix. Diluted cell extract was collected and vortexed for 1 minute. A volume of 100 µl of standard or samples was added to well. Next, the plate was covered with adhesive strip and incubated for 2 hours at room temperature. The wells were then washed four times with washing buffer. The wells were then incubated for 1 hour at room temperature, with 100 µl of active caspase-3 conjugate. Next, the wells were washed four times with washing buffer and incubated with 100 µl substrate solution for 30 minutes at room temperature, and notably protected from light. The reaction was terminated by the addition of 100 µl of diluted hydrochloric acid and the absorbance was read at 450nm. The results were expressed as ng/mg protein.

2.10 Measurement of Active p53 in rat cardiac myocyte Cells

Kit content

Active p53 capture antibody

Active p53 biotin labelled ds oligonucleotide

Active p53 unlabeled ds oligonucleotide

Streptavidin- horseradish peroxidise (streptavidin-HRP)

Other required reagents

Substrate Solution: 1:1 mixture of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine) (R&D Systems)

Stop Solution: 2 N H_2SO_4 (R&D Systems).

Principle

Active p53 concentrations were assayed by DuoSet intracellular (IC) enzyme-linked immunosorbent assay (ELISA) development system. Reagents used in this form of ELISA are sensitive and convenient assays to measure intracellular protein levels in cell lysates. This signal transduction assay makes an excellent alternative to Western blot.

Nuclear extracts from rat cardiac myocyte were incubated with a biotinylated double-stranded (ds) oligonucleotide containing a consensus p53 binding site. P53-ds oligonucleotide complexes were later captured by an immobilied antibody specific for p53. After washing away unbound material, detection utilising streptavidin-HRP was performed. The specificity of the assay was demonstrated with the use of an unlabelled ds competitor oligonucleotide.

Sample preparation

The cells were washed with PBS. Next, cells were solubilised with 400 µl of freshly prepared lysis Buffer A comprising 10 mM HEPES (pH 7.9), 1.5 mM $_{MgCl2}$, 10 mM KCl, 0.5 mM DTT, 2 mM sodium orthovanadate (activated Na3VO4), 3µg/ml aprotinin, 25µg/ml leupeptin, 25 µg/ml pepstatin, 25 µg/ml chymostatin, 0.2 mM phenylmethylsulfonylfluoride (PMSF), and 5 mM sodium fluoride NaF. Then, in a microcentrifuge, the solubilised cells

were centrifuged at 16,000xg for 5 minutes at 4^{o}C. Next, the cytosolic supernatant was discarded and the nuclear pellet was solubilised with 200 µl freshly prepared lysis buffer B, which is composed of 20 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 420 mM NaCl, 0.5 mM DTT, 25% glycerol, 2 mM activated Na_3VO_4, 25 µg/ml leupeptin, 25 µg/ml pepstatin, 25 µg/ml chymostatin, 0.2 mM PMSF, 3 µg/ml aprotinin, and 5 mM NaF. Subsequently, nuclear extract were vortexed for 10 seconds and incubated on ice for 20 minutes. Finally, samples were centrifuged at 16,000 xg for 5 minutes at 4^{o}C.

Procedure

A 96-well plate was coated with 100 µl per well of diluted capture antibody. The plate was covered and was incubated overnight at room temperature and washed three times with washing buffer composed of PBS containing 0.05% Tween 20. Next, it was blocked with 5% bovine serum albumin (BSA) in wash buffer and incubated for 2 hours at room temperature. Following 45 minutes before the end of incubation time, the samples were prepared. To 20 µg of nuclear extract, 3µl of biotin labelled ds oligonucleotide was added in an eppendorf and the final volume was adjusted to 30 µl with lysis buffer B and accordingly incubated at room temperature for 30 minutes. Thereafter, 200 µl of reagent diluent (5% BSA in wash buffer) was added to each sample and mixed well. The wells were then incubated and sealed for 2 hours with 100 µl of sample. The wells were then washed five times with a washing buffer and incubated with 100 µl of streptavidin-HRP diluted in reagent diluent (1:40) for 20 minutes, and protected from light at room temperature. The wells were then washed five times with washing buffer followed by the addition of 100 µl substrate solution and incubated for 20 minutes at room temperature. The reaction was terminated by the addition of 50 µl of stop solution (2 N H_2SO_4) and the absorbance was measured at 450nm.

2.11 Measurement of total p53 in rat cardiac myocyte cells

Kit content

Total p53 capture antibody

Total p53 detection antibody

Total p53 standard

Streptavidin- horseradish peroxidise (streptavidin-HRP).

Other required reagents

Substrate Solution: 1:1 mixture of Colour Reagent A (H_2O_2) and Colour Reagent B (Tetramethylbenzidine) (R&D Systems)

Stop Solution: 2 N H_2SO_4 (R&D Systems).

Principle

Total p53 concentrations were determined through the use of sandwich ELISA. In this method, both phosphorylated and unphosphorylated p53 were bound by immobilised capture antibody specific for p53. A biotinylated detection antibody specific for p53 is used to detect both phosphorylated and unphosphorylated protein using a standard streptavidin-HRP format after washing away unbound material.

Sample preparation

The cells were rinsed twice with PBS. Next, the cells were solubilised with 200 µl of freshly prepared lysis buffer comprising 1 mM EDTA, 0.5% triton X-100, 10 µg/ml leupeptin, 10 µg/ml pepstatin, 3 µg/ml aprotinin, 150 mM NaCl, 10 mM NaF, 1 mM DTT, 20 mM β-glycerophosphate in PBS, pH 7.2–7.4. Subsequently, samples were left on ice for 15 minutes, with samples then centrifuged at 2000g for 5 minutes. The supernatant was transferred to a clean test tube.

Procedure

A 96-well plate was coated with 100 µl of capture antibody diluted to a working solution of 4 µg/ml in PBS. The plate was sealed and incubated overnight at room temperature. Next, the plate was washed three times with a washing buffer comprising PBS containing 0.05% Tween 20. Subsequently, it was blocked with blocking buffer (1% BSA, 0.05% NaN_3, in PBS, pH 7.2–7.4) and incubated for 2 hours at room temperature. The wells were next washed three times with washing buffer and incubated with 100 µl of sample or standard in IC diluent 4 consisting of 1 mM EDTA, 0.5% Triton X-100 in PBS, pH 7.2–7.4. The plate was then covered and left at room temperature for 2 hours. Thereafter, the wells were washed three times with washing buffer and incubated for 2 hours, accordingly sealed at room temperature with detection antibody diluted to a working solution of 1µg/ml in 1% BSA in PBS, pH 7.2–7.4. Following, the wells were washed three times with washing buffer and a freshly prepared streptavidin-HRP diluted in 1% BSA in PBS (1:80). Wells were incubated for 20 minutes at room temperature, and thereby protected from light. Following, the wells were washed three times with washing buffer and 100 µl of substrate solution was added to each well and permitted to develop for 20 minutes at room temperature. Finally, the

addition of 50 µl stop solution (sulphuric acid) stopped the reaction and the absorbance was measured at 450nm.

2.12 Protein estimation (Bio-rad Assay)

In order to determine the protein concentration in cardiac myocyte, 0.5% bovine serum albumin (BSA) was prepared (0.5mg/ml in distilled water). The volume of Biorad dye was diluted in water at a ratio of 1:5. BSA standard for protein estimation were prepared, as can be seen in Table 2.1. From the samples taken, a volume of 10 µl was mixed with 40 µl distilled water and completed to one ml with 950 µl of diluted bio-rad solution. Moreover, standards and samples were measured with spectrophotometer at 595nm.

Table 2.1: Standard curve preparation for protein estimation.

volume of BSA stock (µl)	Volume of dH_2O (µl)	Volume of diluted Bio-rad (µl)	BSA standard
0	50	950	0
10	40	950	5
20	30	950	10
30	20	950	15
40	10	950	20

2.13 Cell culture techniques

Neonatal, ventricular rat cardiac myocytes (LONZA, UK) were maintained in rat cardiac myocyte complete medium (LONZA, UK), and were routinely cultured in T-75 flasks pre-coated with poly-l-lysine. Cells were grown at 37^{o}C under 95% air/ 5% CO_2. Confluent cells were collected via trypsinisation, and passaged every 3 days. Cells used for experiments were between passages 2 and 8.

When cells were confluent, the medium was then removed. Next, the cells were rinsed twice with 10ml of PBS, and 1x trypsin (5-10ml) was added to the cells in T-75 flasks for 5-7 minutes in the incubator. Once the cells detached, 5-10ml of cardiac myocyte complete medium was added to neutralize the trypsin action. Then cell suspension was centrifuged for 5 minutes at 1000 g. The supernatant was discarded, and re-suspension of the pellet in 1ml fresh medium was carried out, and the number of cells per ml determined.

Preparation of freezing medium

The freezing medium was prepared with 10% DMSO in 90% FBS. Briefly, 2.5ml of DMSO in 22.5ml of cold FBS was mixed and kept at 20^{o}C until used. Fresh freezing medium was then prepared or defrosted shortly before use.

Freezing of cells

From one confluent 75 cm^3 flask, two cryotubes were frozen. Approximately 3.5 x 10^5 cells per cryovial were frozen. Cryovials containing cells were kept at –20^{o}C for 30 minutes, at –

80^{o}C overnight, and thereafter transferred to the fume phase of liquid nitrogen. Cells were ultimately stored in liquid nitrogen for an indefinite time

Thawing of cells

From liquid nitrogen, frozen cryotubes of cells were taken and disinfected by spraying with 70% ethanol. The pressure inside the tubes was liberated inside a laminar flow hood, and the frozen cells were then slowly defrosted at 37°C into a T-75 flask containing pre-warmed complete medium. Subsequently, the resulting cell suspension was transferred and then incubated at 37°C.

2.14 Cell counting

With the aid of a Coulter counter, cells were counted. Briefly, 0.1ml of cell suspension was added to 20ml isotone solution in a counting chamber, mixed and counted at least three times. Cells in suspension were either subcultured or frozen in liquid nitrogen. Cell numbers correspond to:

X (cell number) x (20/0.5) = X x 40 (dilution factor)

Notably, this equation was used when 0.5ml of trypsinised cell suspension was added to 20ml of isoton solution and the number of cells was counted using the Coulter counter.

2.15 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay

Approximately 1 x 10^3 to 1 x 10^{10} rat cardiac myocyte were seeded in 100µl of rat cardiac myocyte complete medium in 96-well plates. The plate was incubated at 37°C in a humidified, 5% CO_2 atmosphere, and permitted to attach for approximately 4 hours.

Following cell attachment, a different treatment was applied for 24 hours. In order to determine cell number in 96-well plate, the cell proliferation of cultured cells was quantified using the MTS kit (Cell Titer 96® AQueous One Solution cell proliferation assay). The assay uses a solution of MTS (Owen's reagent, a tetrazolium compound and phenazine methosulfate, an electron coupling reagent), and was carried out according to the manufactureer's instructions. In brief, 20µl of Cell Titer 96® AQueous One Solution reagent was added to each 96-well plate containing 100µl of cell suspension. Subsequently, cells were incubated for 2 hours in a humidified, 5% CO_2 atmosphere, and the absorbance was then accordingly recorded at 490nm using an ELISA plate reader. The proliferation of cultured cells was estimated by determining the mean absorbance ± SD of three experiments.

2.16 Detection of apoptosis with propidium iodide (PI) /4,6-diamidino-2-phenylindole (DAPI) staining

Glass cover slips were soaked in pure ethanol 100% for 20 minutes. Subsequently, cover slips were placed in 24 well plate and left to air dry inside the biosafety cabinet. Rat cardiac myocyte $5X10^4$ were seeded in 24 well plate that was previously coated with the substrate, poly-l-lysine. After 24 hours, cell attachment staurosporine 0.25 µM was permitted, and different drug treatments were added in a fresh complete medium for 24 hours. One hour before the end of the experiment, propidium iodide 10 µg/ml was added and incubated with protection from light. Afterwards, the medium was then discarded and cells washed twice with PBS. Next, the cells were fixed in 4% paraformaldehyde (50 µl/well) and incubated at room temperature for 20 minutes. Subsequently, the paraformaldehyde was discarded and cells rinsed with PBS. Finally, one drop of mounting medium with DAPI (Vectashield mounting medium for fluorescence with DAPI) was placed on a slide and the cover slip was

put upside down on the mounting medium, with the slides kept at 4^{o}C. The next day, slides were examined using fluorescent microscopy.

Molecular biology techniques

2.17 RNA extraction for polymerase chain reaction (PCR) array and real time PCR

The relative levels of mRNA expression for a number of genes involved in oxidative stress were determined by real-time PCR and microarray. Qiagen column extraction system was used to extract total RNA. Rat cardiac myocyte were cultured on 6 well plate to confluence before the addition of AGE (100 µg) or DOX (10 µM) or both of them for 24 hours. The cells were washed twice with PBS then 750 µl of trypsin 1x was added per well and then incubated for 2–5 minutes. Next, when the cells detached, 750µl of cardiac myocyte complete medium was added to counteract the trypsin action. Next, the cell suspension was centrifuged for 5 minutes at 1000g. The supernatant was discarded, and re-suspention of the pellet in 500 µl PBS was carried out and centrifuged at 1500g for 5 minutes. Later, supernatant was discarded and pellet stored in –80^{o}C until analysis. To the pellet, 1 volume (350 µl) of 70% ethanol was added, and mixed well via pipetting. The samples were then transferred to an RNeasy MinElute spin placed in a 2ml collection tube, and accordingly centrifuged (8500g for 30 seconds). During this process, RNA bound to the RNeasy silica gel membrane. A volume of 350 µl of RW1 buffer (containing a guanidine salt) was added to the column and centrifuged (8500g for 30 seconds). A DNase incubation combination was prepared by adding 10 µl DNase 1 to 70 µl buffer RDD. Next, the total volume was pipetted directly into the RNase silica gel membrane and incubated at room temperature for 15 minutes in order to digest and remove any traces of DNA. After that, the column was rinsed

clean of DNase and other contaminants by adding 350 µl of RW1 buffer and centrifuged for 30 seconds at 8500g. The column was then placed into a new 2 ml collection tube, and 500 µl buffer PRE was added to wash the colum by centrifuging for 30 seconds at 8500g. Following this, 500 µl of 80% ethanol was added to the RNeasy MinElute column and centrifuged for 2 minutes at 8500g in order to rinse the column. The column was then transferred to a new 2 ml collection tube and centrifuged at full speed for 5 minutes with the lids open so as to completely dry the column and ensure that no ethanol was carried over during RNA elution. Afterwards, the column was placed into a new 1.5 ml collecting tube, and 30 µl of RNase-free water was pipetted directly to the centre of the spin column membrane and centrifuged for 1 minute at full speed in order to elute the RNA. Following, RNA samples were stored at –70^{o}C.

Measurement of RNA concentration

The concentration of known volume of RNA was calculated through measuring absorbance at 260nm and 280nm using an ultrospec 2000 spectrophotometer, which was blanked against water. Absorbance ratio A_{260}/A_{280} was calculated to assess RNA quality. An absorbance ratio of 1.8-2 indicates pure RNA.

2.18 cDNA synthesis using RT^{2} first Strand kit

An amount of 2.4 µg of RNA was used from each sample. In a sterile PCR tube, 2µl GE (5X g DNA elimination buffer) was added to 2.4 µg total RNA and was made up with water (H_2O) to a final volume of 10 µl and then mixed gently with a pipettor. Next, the PCR tubes were incubated at 42^{o}C for 5 minutes, followed by immediate chilling on ice for 2 minutes. Then, RT cocktail was prepared which composed of 4 µl BC3 (5X RT buffer), 1 µl P2 (primer and external control mix), 2 µl RE3 (RT enzyme mix 3) and 3 µl water to get a final

volume of 10 µl for one reaction. A volume of 10 µl of RT cocktail was added to each 10 µl genomic DNA elimination mixture and mixed gently with a pipettor. Subsequently, the tubes were incubated at 42^{o}C for exactly 15 minutes, and the reaction was immediately stopped by heating at 95^{o}C for 5 minutes. To each 20 µl of cDNA reaction, 91 µl of water was added and mixed well. Finally, the first strand cDNA synthesis reaction was stored at –20^{o}C.

2.19 Rat oxidative stress and antioxidant defence PCR array

The most sensitive and reliable method for gene expression analysis is real-time reverse transcription (RT) PCR. The RT^{2} Profiler PCR Array takes advantage of real-time PCR performance and combines it with the ability of microarrays in order to detect the expression of many genes simultaneously.

The procedure of PCR Array starts by converting RNA samples into first strand cDNA the template for the polymerase chain reaction utilising RT^{2} First Strand Kit. Subsequently, cDNA template was mixed with RT^{2} qPCR Master Mixes. The mixture was then pipetted into each well of the plate containing pre-dispensed gene specific primer sets. PCR was then performed, and finally, the relative expression was determined with Stratagene MX3000p and the DDCt method.

The expression of 84 genes relating to oxidative stress was achieved with the use of the rat oxidative stress and antioxidant defence RT² Profiler™ PCR Array. The genes included in this array are peroxidases, such as glutathione peroxidase (Gpx) and peroxiredoxins (Prdxn). The genes involved in ROS metabolism, such as oxidative stress responsive genes, those involved in superoxide metabolism, such as superoxide dismutases (SOD), and oxygen-transporter genes are also included in this array. The expression of a specific altered gene relating to oxidative stress in this array was further tested with real-time PCR.

2.20 Quantitative real-time reverse transcription PCR (RT^2 qPCR)

The presence and number of copies of a specific DNA sequence in a given sample can be measured with quantitative real-time PCR. It is known as real-time because, unlike normal PCR, the DNA is quantified after each amplification cycle. By combining real-time PCR with reverse trancriptase PCR (RT-PCR), quantification of low-abundance mRNA using fluorescent dye is achievable. Furthermore, relative gene expression in a particular cell at a particular time can be measured.

Real-time PCR

Assessment of the expression of oxidative stress genes was quantified using RT^2-qPCR. Semi-quantitative reverse transcription polymerase chain reaction (Semi-qRT-PCR) was applied to measure the relative quantities of oxidative stress gene transcripts in rat cardiac myocytes. Moreover, the total RNA was extracted, as described in Section 2.17, and was transformed directly to cDNA synthesis, as described in Section 2.18. The primers for oxidative stress genes and housekeeping genes were ordered from SA Bioscience, USA. cDNA was amplified using RT^2-qPCR with fluorescence using SYBR Green, with fluorescence binding to all newly synthesized double-stranded DNA. Measurement of the increase in the fluorescence intensity allowed determination of the initial concentration. Fluorescence is determined at the end of each PCR cycle and increases exponentially as the reaction progress. The threshold cycles ($C_{t)}$ for each reaction is calculated based on the point at which a statistically significant increase in the amount of PCR product is detected. The C_t inversely indicates the number of target sequences present in each sample prior to amplification (Higuchi *et al.*, 1993).

For each condition, reactions were conducted in triplicate and the mean C_t subsequently calculated. The amplification of a known mRNA concentration in serial dilutions was measured in order to determine the sensitivity of the expression. For all primers, PCR efficiency was assessed.

Amplification of cDNA aliquots was carried out in 25 µl reaction volume containing 1 µl RT^2 First Strand cDNA, and 1 µl of gene specific 10 µM PCR primer pair stock with 12.5 µl of RT^2 SYPR Green qPCR master mix and 10.5 µl H_2O. The thermal cycle conditions consist of an initial denaturation step at 95^{o}C for 10 minutes, followed by 40 cycles at 95^{o}C for 15 seconds, 60^{o}C for 1 minute, and 72^{o}C for 30 seconds. The assessment of specificity of the amplified product was then performed through the evaluation of dissociation curves which showed product melting points.

2.21 Reverse transcription-PCR (RT-PCR)

The cDNA generated with the first strand kit was used in RT-PCR. In a 0.25ml PCR tube, cDNA 1 µl, primer 1 µl, and water 10.5 µl was mixed and then completed to a final volume of 25 µl with 12.5 µl of buffer using AmpliTaq PCR master mix kit (Applied Biosystem). PCR was accomplished using a PTC-200 programmable thermal controller (MJ Research, USA). The samples were denaturated at 95^{o}C for 10 minutes, followed by 38 cycles of denaturation at 95^{o}C for 30 seconds, primer annealing at 60 ^{o}C for 1 minute, and DNA extension at 72^{o}C for 1 minute.

2.22 Agarose gel electrophoresis of DNA

PCR products were electrophoresed at 100 V through 2% agarose gel in Tris-borate-EDTA (TBE) electrophoresis buffer. A volume of 10 µl from sample was mixed with 10 µl of 2x

DNA dye and loaded on the agarose gel. The gels were then stained in a solution composed of 10 µl ethidium bromide in 100ml distilled water for 30 minutes. Thereafter, bands were visualised on an ultraviolet tranilluminator and photographed using Gene snap programme from G Box.

2.23 Statistical analysis

Statistical analyses were performed using the statistical software package SPSS (version 15; SPSS Inc., Chicago). Results were expressed as the mean ± standard error of the mean (SEM). The experimental data were statistically analysed using one-way analysis of variance (ANOVA) followed by the LSD test. The unpaired student t-test was used for two group statistical analysis. The level of significance was set at $p < 0.05$.

Chapter 3 Effect of aged garlic extract against doxorubicin–induced cardiotoxicity in rats and antitumor activityof doxorubicin in mice

3.1 Introduction

The clinical use of DOX is restricted by the dose-dependent side effects of cardiotoxicity, which may lead to irreversible cardiomyopathy, and ultimately heart failure (Shan *et al.*, 1996). The cardiotoxic effects of DOX may occur promptly after a single dose, or several weeks to months after repetitive DOX administration. A number of explanations account for the DOX cardiotoxicity; free radical production, calcium overloading, mitochondrial dysfunction and peroxynitrite formation have all been proposed as mechanisms (Olson and Mushlin, 1990; Mihm *et al.*, 2002; Denicola and Radi, 2005; Mukhopadhyay *et al.*, 2009).

The semiquinone form of DOX is a toxic, short-lived metabolite that interacts with molecular oxygen and starts a cascade of reactions, producing ROS (Gilleron *et al*., 2009; Thorn *et al*., 2010). The free radical hypothesis is well accepted and documented. Owing to the proposed role of free radicals in DOX cardiotoxicity, compounds with antioxidant activity may protect against DOX-induced toxicities in the heart (Siveski-Iliskovic *et al.*, 1995; Qin *et al.*, 2008).

Aged garlic extract has been reported to have powerful antioxidant and free radical scavenging properties (Borek, 2001; Drobiova *et al.*, 2009) and may prove useful as a protectant against DOX-induced cardiotoxicity.

The current study included four groups of rats, control, AGE, DOX, and AGE+DOX treated rats.This study aimed to investigate the ability of AGE in terms of protecting against DOX-induced cardiotoxicity. The specific objectives were:

To determine serum CPK, LDH, MDA, and TAS in the four different groups of rats

To study histopathological changes in rats treated with DOX, AGE or both.

To evaluate the effect of AGE on the antitumour activity of DOX by measuring survival of mice bearing Ehrlich ascites carcinoma (EAC) tumour and determination of effect of AGE-pre-treatment on DOX uptake in EAC-cells

To determine tissue distribution of DOX in the presence and absence of AGE in the plasma, heart, liver, and kidneys of mice at different time points.

3.2 Materials and Methods

The AGE used in following experiments contained 28.6% extracted solids (286 mg/ml), and S-allyl cysteine, the most abundant water-soluble compound in AGE was present at 1.47 mg/ml.

Animal experiments part 1

Wistar albino rats (8–10 weeks of age, 180–200g body weight) were obtained from King Fahad Medical Research Center, King Abdul Aziz University, Jeddah, Saudi Arabia. The animals were conditioned for one week at room temperature. A commercial balanced diet and tap water, was provided throughout the experiment *ad libitum*. This study was approved by the ethics committee of King Abdul Aziz University Medical Faculty.

Twenty-four male Wistar rats were divided into four equal groups, each comprising six animals, and housed in a room with regular light/dark cycle with free access to food and water.

Two groups namely Group I and Group II were used as a control. Group I received normal saline, intraperitonealy (i.p.), and distilled water per oral (p.o.). Group II received AGE 250 mg/kg orally for 28 days. Group III received a single i.p. dose of DOX (25 mg/kg) on day 27, following the successive administration of distilled water (0.5ml orally) (Venkatesan, 1998). Group IV received a single i.p. dose of DOX (25 mg/kg) on day 27, following the successive administration AGE (250mg/kg orally).

At the end of the experimental period (29 days), 48 hours, after DOX injection, rats were anesthetised and blood samples were collected from the ophthalmic artery in the orbital rim prior to sacrifice. Serum was separated and heart specimens were fixed in10% formalin for histopathological examination. Samples were then analysed for concentrations of serum CPK, LDH, MDA and TAS.

The concentrations of serum total CPK were measured by the method described in Section 2.3, serum LDH was assayed by the method described in section 2.4, the concentration of plasma and heart MDA was measured by the method described in Section 2.6, the concentrations of TAS in the serum were measured by the method described in Section 2.7, histopathological changes were examined as described in Section 2.5.

Animal experiments part 2

Female Swiss albino mice (8 weeks of age, 20–25g body weight) were obtained from King Fahad Medical Research Center, King Abdul Aziz University, Jeddah, Saudi Arabia. The

animals were acclimatised for one week at room temperature. A commercial balanced diet and tap water were given throughout the experimental period, *ad libitum*. A line of EAC cells was supplied by Prof Abdel-Moneim and maintained in our laboratory by weekly i.p. transplantation of 2.5 X 10^6 cells/ mouse. This study was approved by the ethics committee of King Abdul Aziz University Medical Faculty.

Evaluation of antitumour activity

The effect of AGE on the antitumour activity of DOX was evaluated using the method of Donenko *et al.* (1991) with slight modification. In brief, EAC cells were injected i.p. into forty female Swiss albino mice (2.5 x 10^6 cells/mouse). Subsequently, 24 hours later, mice were equally divided into four groups. In Group 1, mice were administered distilled water p.o. daily for six days and received saline 0.2 ml i.p. every other day for a total of 3 doses and served as the control group. In Group 2, mice were administered AGE 2860 mg/kg p.o. once daily for six days and served as the aged garlic group (Wang *et al.*, 1999). In Group 3, mice were injected with DOX (2 mg/kg i.p.) every other day for a total of 3 doses and served as the DOX group. In Group 4, mice were administered AGEs 2860 mg/kg p.o. once daily for six days before DOX injection (2 mg/kg i.p.) every other day for a total of 3 doses served as DOX-aged garlic group. Average survival time for mice and long-term survivors are defined as the mice who survived to the end of experiment (90 days) without any apparent evidence of tumour cell growth.

Effect of aged garlic extract pre-treatment on doxorubicin uptake in EAC-cells

Ehrlich ascites carcinoma cells (EAC) were inoculated as described above at 2.5 x 10^6 cells/mouse. Twenty four hours later, AGE (2860 mg/kg p.o.) or an equal volume of saline was administered once per day for 6 days. On the 6^{th} day, DOX was injected i.p. in a single

dose (15 mg/kg). Six hours after DOX therapy, EAC-cells were withdrawn from each group, counted, homogenised and extraction of DOX was performed according to the method of Bachur *et al.* (1970). The concentration of DOX was measured spectrofluorometrically using a Perkin Elmer fluorescence spectrometer LS55 with excitation and emission wavelengths of 470 and 585nm respectively.

Tissue distribution of doxorubicin in the presence and absence of aged garlic extract

Forty-eight female Swiss albino mice (20–25g) were inoculated with 0.2 ml of (2.5×10^6) EAC i.p. Subsequently, 24 hours later, animals were divided into two groups (24 mice each): Group I was injected with DOX (15 mg/kg i.p.) 10 days following the administration of 0.2 ml distilled water orally; Group II was injected with DOX (15 mg/kg i.p.) 10 days after administration of AGE 2860 mg/kg orally.

At the end of the experiment, mice were anesthetised and blood samples were collected at 24, 48, 72 and 120 hours following treatment from ophthalmic artery in the orbit rim prior to sacrifice. Serum was separated and the heart, liver and kidneys of each animal were dissected and used for determination of DOX according to the method of Bachur *et al.* (1970).

3.3 Results

Animal experiments part 1

Serum total creatine phosphokinase

The mean serum CPK for the four groups of male Wistar rats is shown in Figure 3.1. The results show that the mean ± SEM of the concentration of serum CPK was 383.5 ± 71.944

U/L for control rats, 457.66 ± 41.38 U/L for AGE treated rats, 1094 ± 158.43 U/L for DOX treated rats, 666 ± 25.82 U/L for AGE-DOX treated rats. There was no significant difference in serum CPK concentration between control and AGE treated rats. Nevertheless, the concentration of serum CPK was significantly ($p<0.001$) higher in DOX treated rats than control rats. This increase was significantly ($p<0.05$) lower with AGE-pre-treatment.

Serum lactate dehydrogenase

Figure 3.2 displays the mean ± SEM for serum LDH concentrations for the four groups. The mean ± SEM of the concentrations of serum LDH were 2414.33 ± 420.89 U/L for control rats, 3007.83 ± 278.19 U/L for AGE treated rats, 4325 ± 599.63 U/L for DOX treated rats, and 3018,17 ± 167.266 for AGE-DOX treated rats. There was a significant ($p<0.05$) increase in serum LDH concentration only in DOX treated rats as compared to control and AGE-DOX treated rats. This increase was significantly lower with AGE pre-treatment ($p<0.05$).

Plasma concentrations of malondialdehyde

Figure 3.3 shows the calibration curve of MDA. Plasma concentrations of MDA are presented in Figure 3.4. The mean ± SEM of the concentrations of plasma MDA were 0.657 ± 0.436 µM for control rats, 5.53 ± 3.24 µM for AGE treated rats, 11.94 ± 1.96 µM for DOX treated rats, and 5.54 ± 1.88 µM for AGE-DOX treated rats. There was a significant ($p<0.05$) increase in plasma MDA concentration only in DOX treated rats as compared to control and AGE-DOX treated rats. Aged garlic extract pre-treatment significantly reduced the rise in plasma MDA, which was caused by DOX ($p<0.05$).

Heart concentrations of malondialdehyde

Heart concentrations of MDA are shown in Figure 3.5. The mean ± SEM were 20.42 ± 3.95 µM for control rats, 23.23 ± 2.95 µM for AGE treated rats, 38.77 ± 1.02 µM for DOX treated rats, and 22.77 ± 4.79 µM for AGE-DOX treated rats. There was a significant ($p<0.001$) increase in heart MDA concentration only in DOX treated rats when compared with control. Furthermore, there was a significant increase in heart MDA concentration compared with AGE-DOX treated rats ($p<0.05$). Aged garlic extract pre-treatment prevented DOX-induced increased MDA production in rat heart.

Serum total antioxidant status

Antioxidant activity was assessed by measuring TAS. The mean serum TAS for the four groups of male Wistar rats is shown in Figure 3.6. The results showed that the mean ± SEM for serum TAS was 1.22 ± 0.058 mmol/L for control rats, 1.36 ± 0.01 mmol/L for AGE treated rats, 1.09 ± 0.05 mmol/L for DOX treated rats, 1.22 ± 0.01 mmol/L for AGE-DOX treated rats. As expected, there was a significant increase in TAS ($p < 0.05$) in AGE treated rats as compared to control rats. A significant ($p<0.05$) decrease in TAS was observed with DOX treated rats as compared to control and AGE-DOX treated rats.

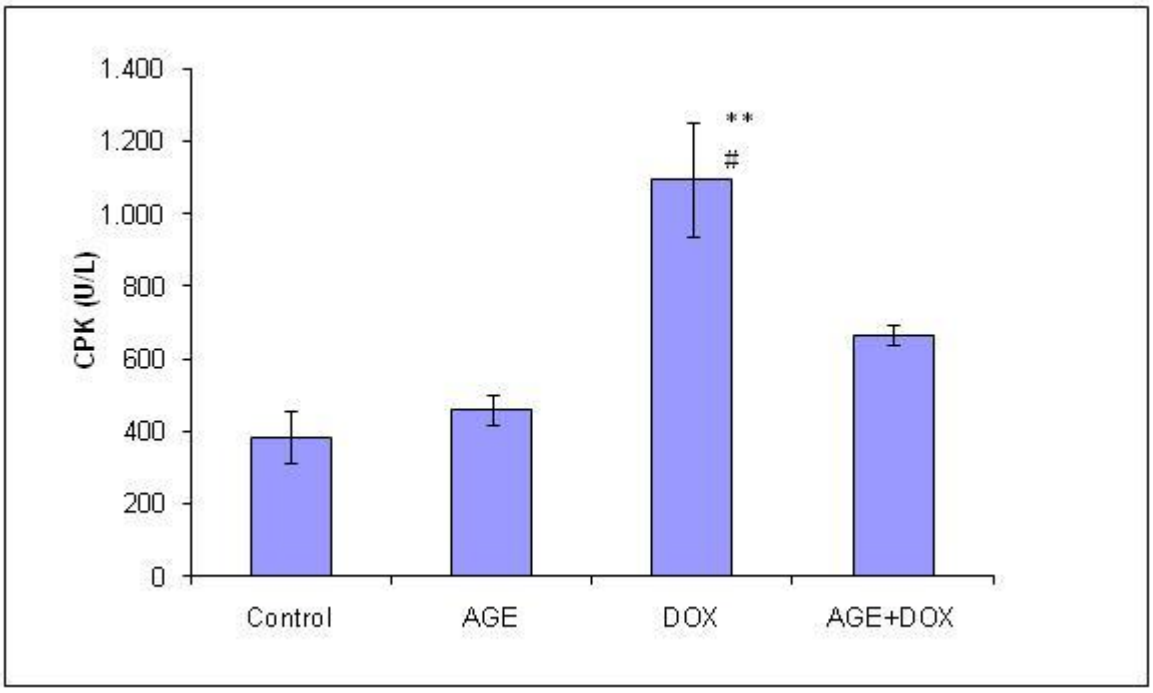


Figure 3.1: Effect of DOX (25 mg/kg) alone or after pretreatment with AGE on the activity of cardiac enzyme CPK. Data are presented as mean ± SEM (n = 6). ** Significantly different from control ($p < 0.001$), # significantly different from DOX ($p<0.05$). No significant difference between between AGE and AGE+DOX ($p>0.05$, one way ANOVA with LSD post test).

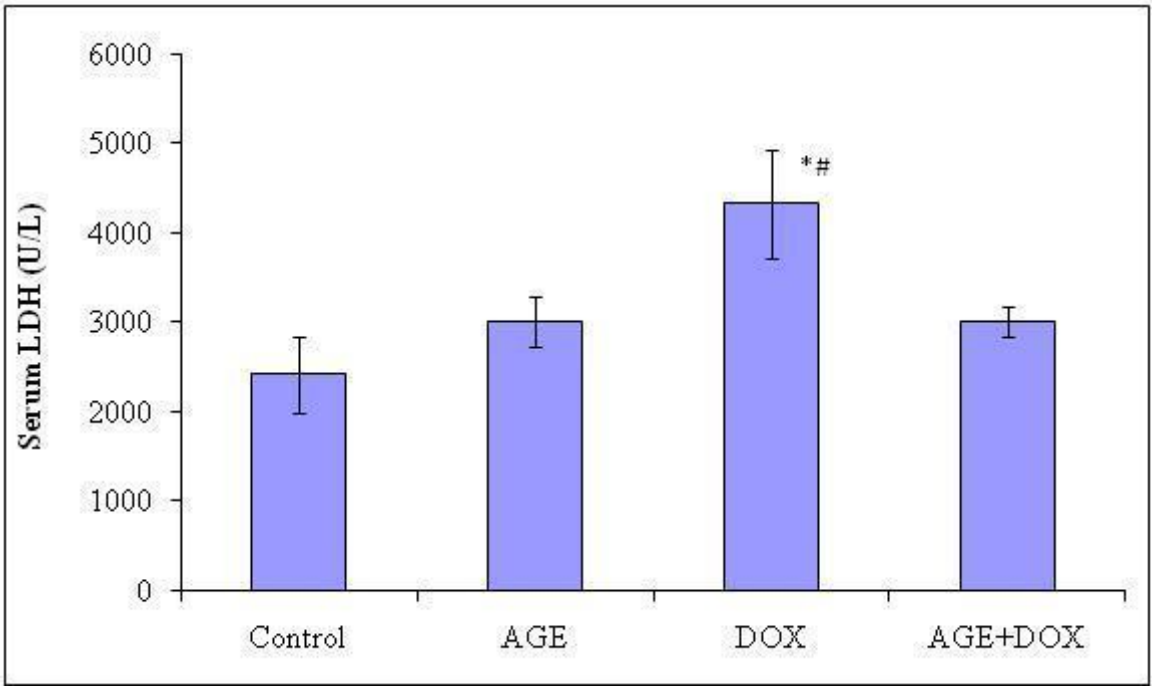


Figure 3.2: Effect of DOX (25 mg/kg) alone or after pretreatment with AGE on the serum LDH activity (U/L) of male Wistar rats. The values are presented as mean ± SEM (n=6). * Significantly different from control ($p < 0.05$), # significantly different from AGE+DOX ($p<0.05$). No significant difference between between AGE and AGE+DOX ($p>0.05$, one way ANOVA with LSD post test).

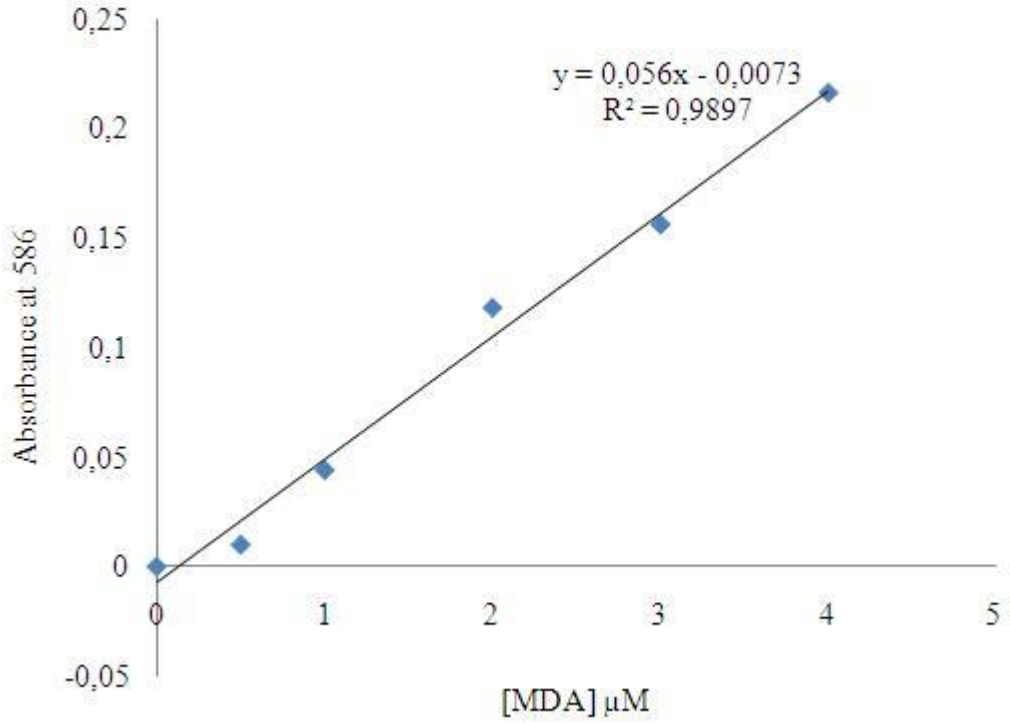


Figure 3.3: The calibration curve of MDA. This curve is typical of 3 different experiments

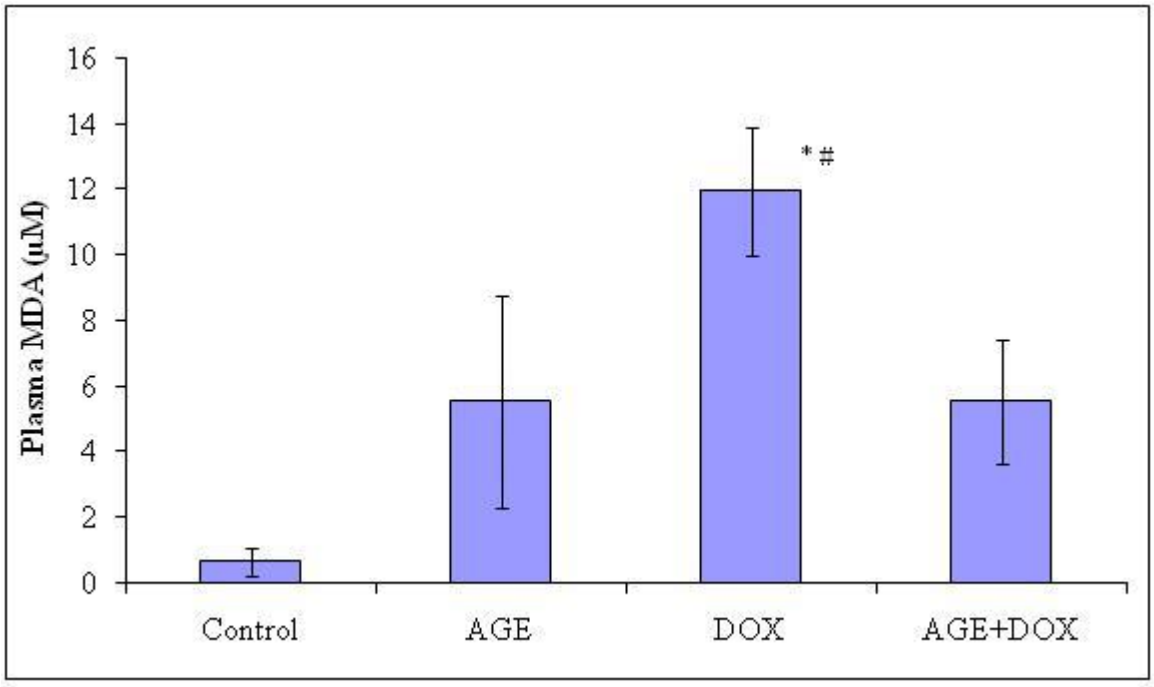


Figure 3.4: Effect of DOX (single dose 25 mg/kg, i.p.) alone and after pre-treatment with AGE (250 mg/kg, p.o.) on plasma malonyldialdehyde (MDA) activity (µM/g tissue) of male Wistar rats. Results are expressed as means ± SEM (n = 6). * Significantly different from control ($p < 0.05$), # Significantly different from AGE+DOX ($p<0.05$). No significant difference between between AGE and AGE+DOX ($p>0.05$, one way ANOVA with LSD post test).

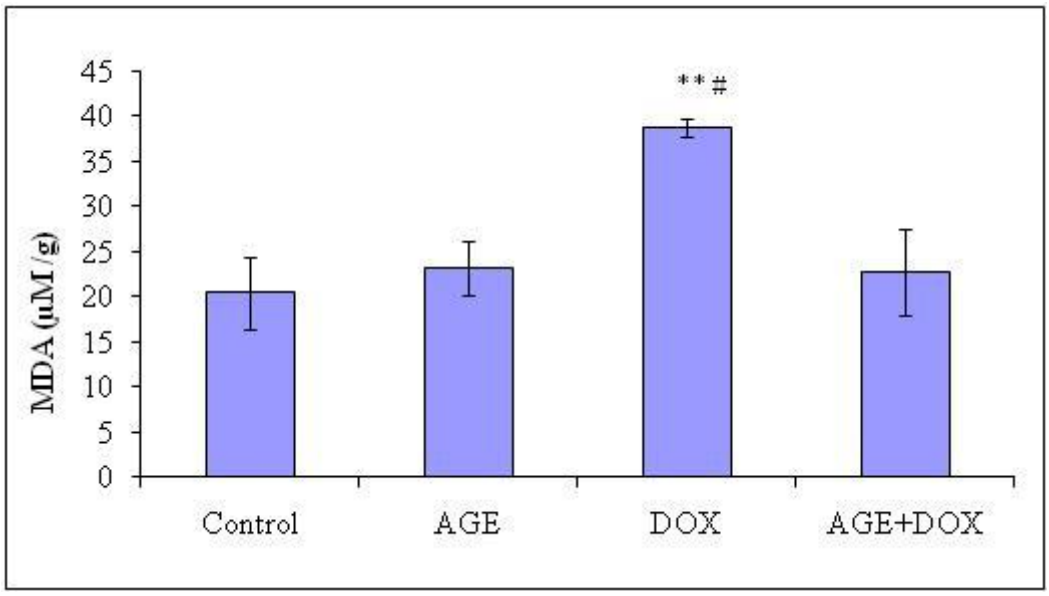


Figure 3.5: Effect of DOX on heart homogenate malonyldialdehyde (MDA) activity (µM/g tissue; single dose 25 mg/kg, i.p.) alone and after pre-treatment with AGE (250 mg/kg, p.o.) in male Wistar rats. The values are presented as mean ± SEM (n=6). ** Significantly different from control ($p < 0.001$), # significantly different from AGE+DOX ($p<0.05$). No significant difference between between AGE and AGE+DOX ($p>0.05$, one way ANOVA with LSD post test).

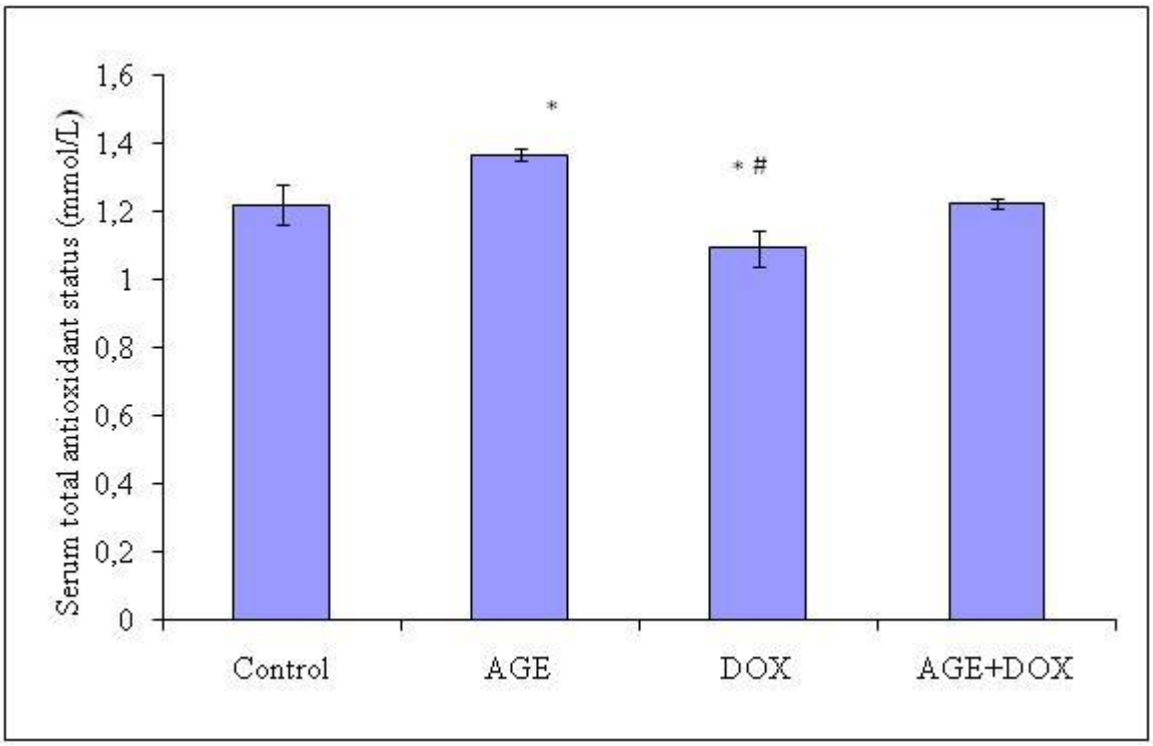


Figure 3.6: Effect of DOX on serum total antioxidant status (TAS) activity (mmol/L; single dose 25 mg/kg, i.p.) and /or AGE pre-treatment (250 mg/kg, p.o.) in male Wistar rats. The values are presented as mean ± SEM (n=6). * Significantly different from control ($p< 0.05$), # significantly different from AGE+DOX ($p<0.05$). No significant difference between between AGE and AGE+DOX ($p>0.05$, one way ANOVA with LSD post test).

Structural and ultrastructural changes in rat heart

In the four groups of rats namely, control, AGE, DOX, AGE+DOX treated rats, light and electron microscopic analyses of left ventricle were carried out. Figure 3.7 show normal cardiac myocytes, oval central nuclei and thin wall blood capillaries Light microscope examination of DOX-treated heart stained with hematoxylin and eosin, showed periarterial fibrosis, loss of striation and increase in inflammatory cells as compared with control rats (Figure 3.8). The histopathologic changes induced by DOX were less in AGE+ DOX treated rats. Rats treated with AGE + DOX showed normal appearance of nuclei and striation. There was noticeable increase in blood vessels in the heart of AGE+DOX treated rats (Figure 3.9). Furthermore, there was extensive cardiac damage in DOX-treated rats as shown by the electron microscopic study. Figures 3.10 and 3.11 demonstrate normal shape mitochondria, tubular cristae and glycogen deposition as shown under the electron microscopic examination of control and AGE-treated rats, respectively. Rats treated with DOX displayed mitochondrial degeneration and swelling, intracytoplasmic vacuolization, and focal myofilament disarrangement (Figure 3.12) while the pre-treatment of rats with AGE caused remarkable reduction in the cellular damage (Figure 3.13). Similar to light microscopic observations, there was vascular congestion and increase in blood vessels with AGE pre-treatment.

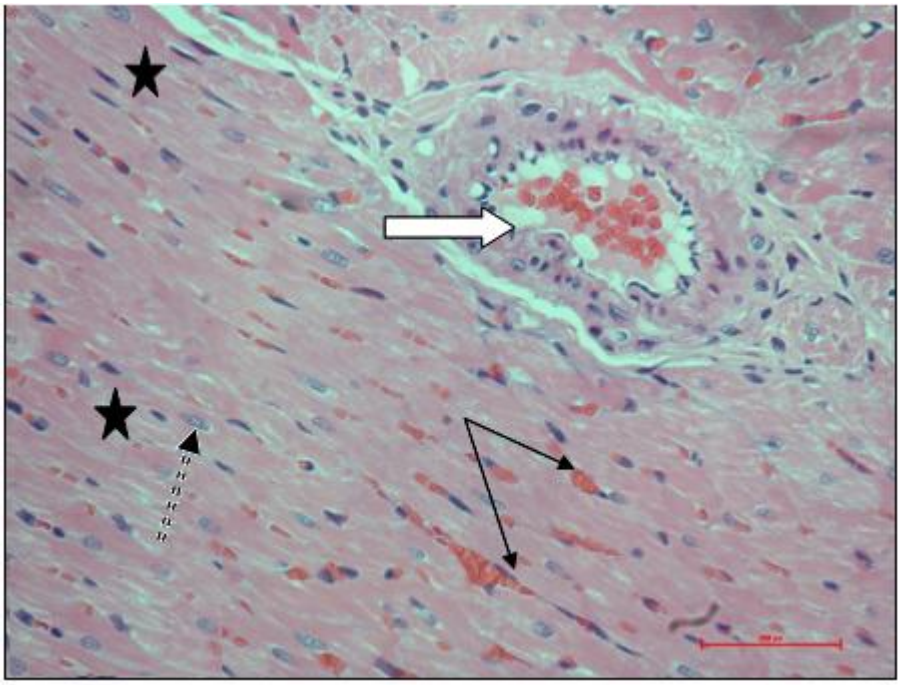


Figure 3.7: Photomicrographs of normal heart tissue. Histological section from the left ventricle showing normal cardiomyocytes (black stars), with oval vesicular central nuclei (dotted black arrow), together with thin wall blood capillaries (thick white arrow), and a branch of the coronary artery were seen amongst the cardiac fibres (thin black arrow) (H&E x40).

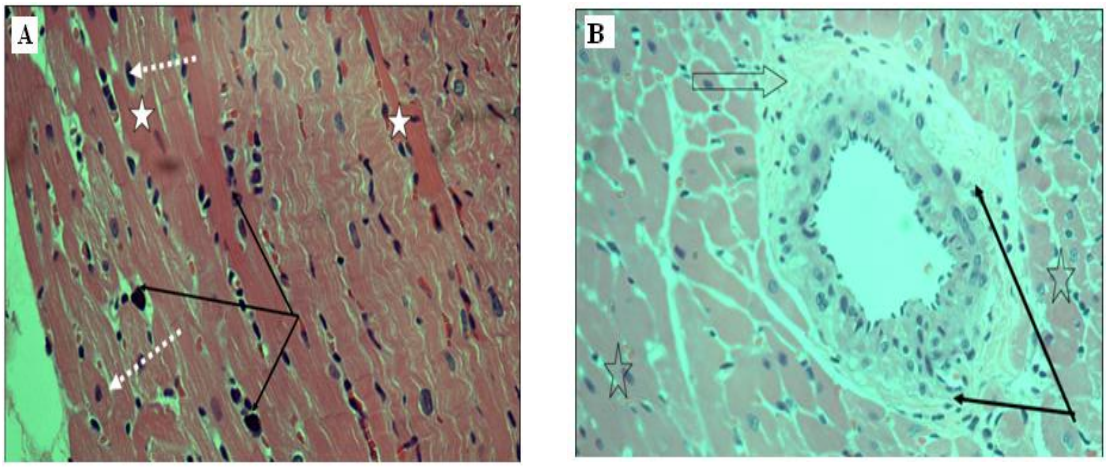


Figure 3.8: Light micrograph of DOX treated rat heart. (A) Dark small nuclei (black arrows and white dashed arrows). (B) Periarterial fibrosis (thick arrow), and increase in inflammatory cells (black arrows) (H&E x 40).

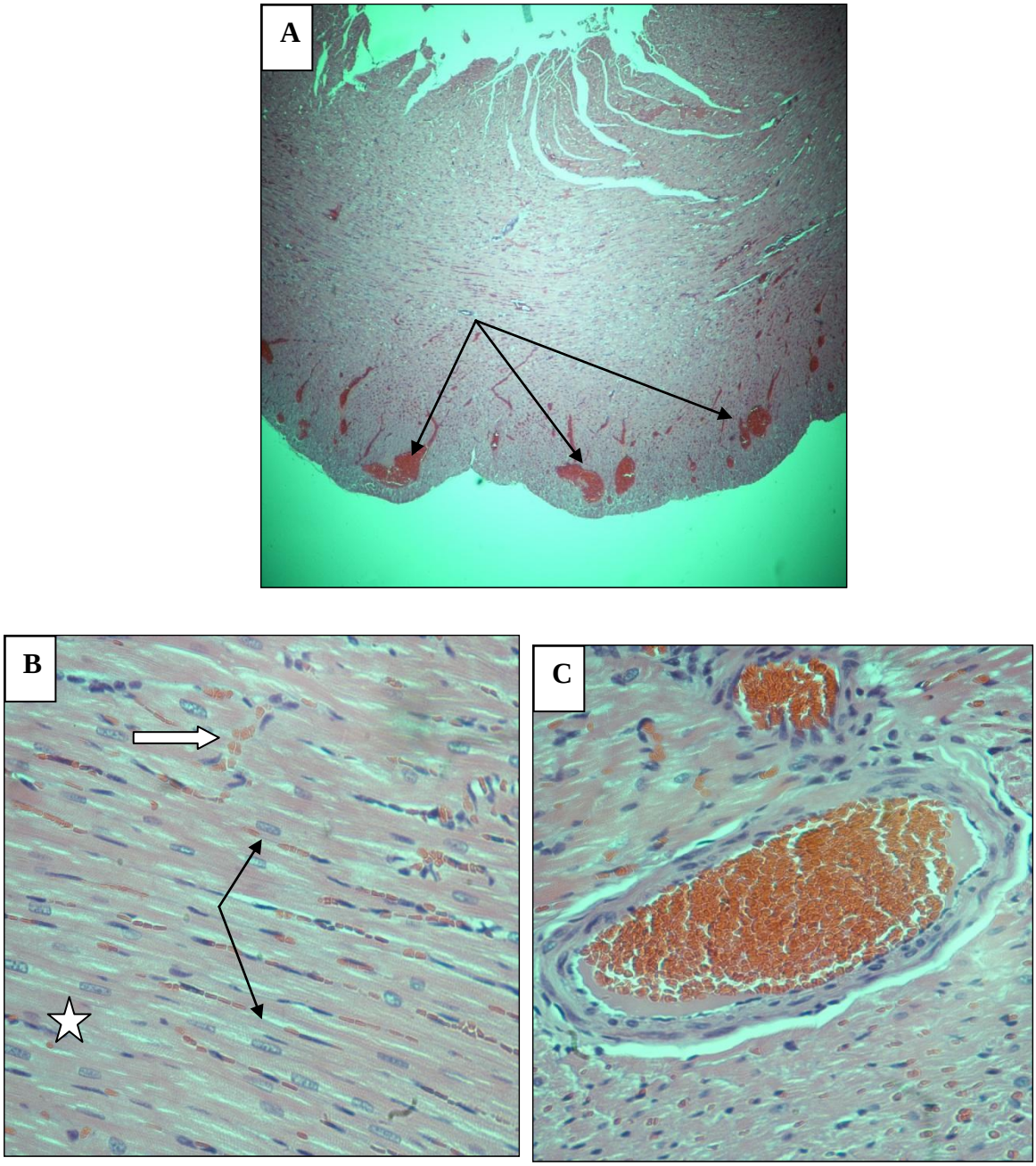


Figure 3.9: Photomicrographs from AGE + DOX treated cardiac tissue. (A) There was increase in sub pericardial vascularity (arrows) (H&E x 20). (B) Normal cardiomyocytes with normal appearance of nuclei and striation (star). The nuclei of the cells are oval vesicular and central. (C) Normal but dilated congested vessels among cardiac fibres were seen (H&E x40).

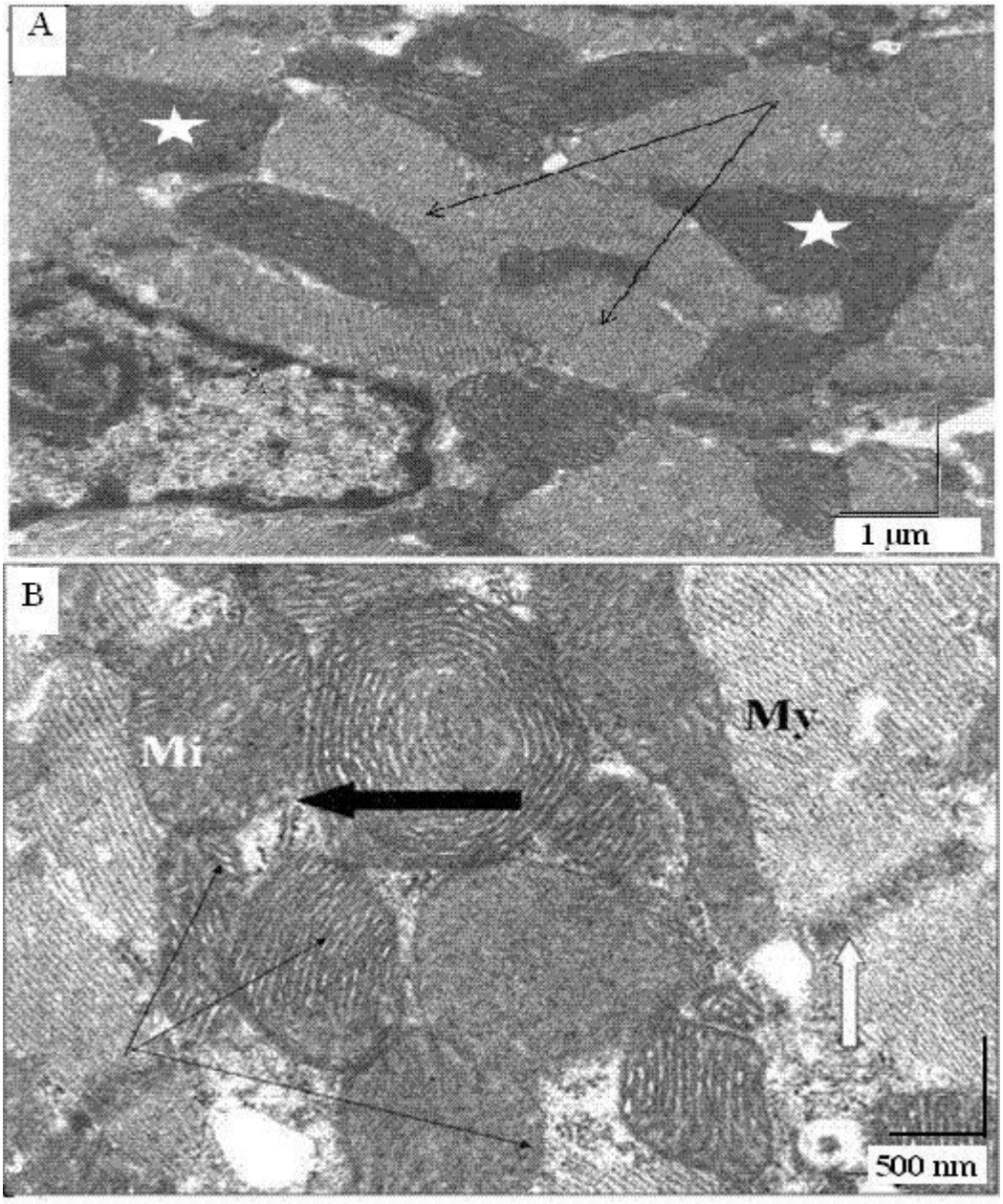


Figure 3.10: Electron photomicrograph of rat heart in control animals. (A) Normal mitochondria (stars) and myofibrils (arrows). (B) Normal heart tissue showing the numerous mitochondria (Mi) with tubular cristae (thick dark arrow) and myofibrils (MY). Glycogen granules (thin black arrows). Z line (white arrow).

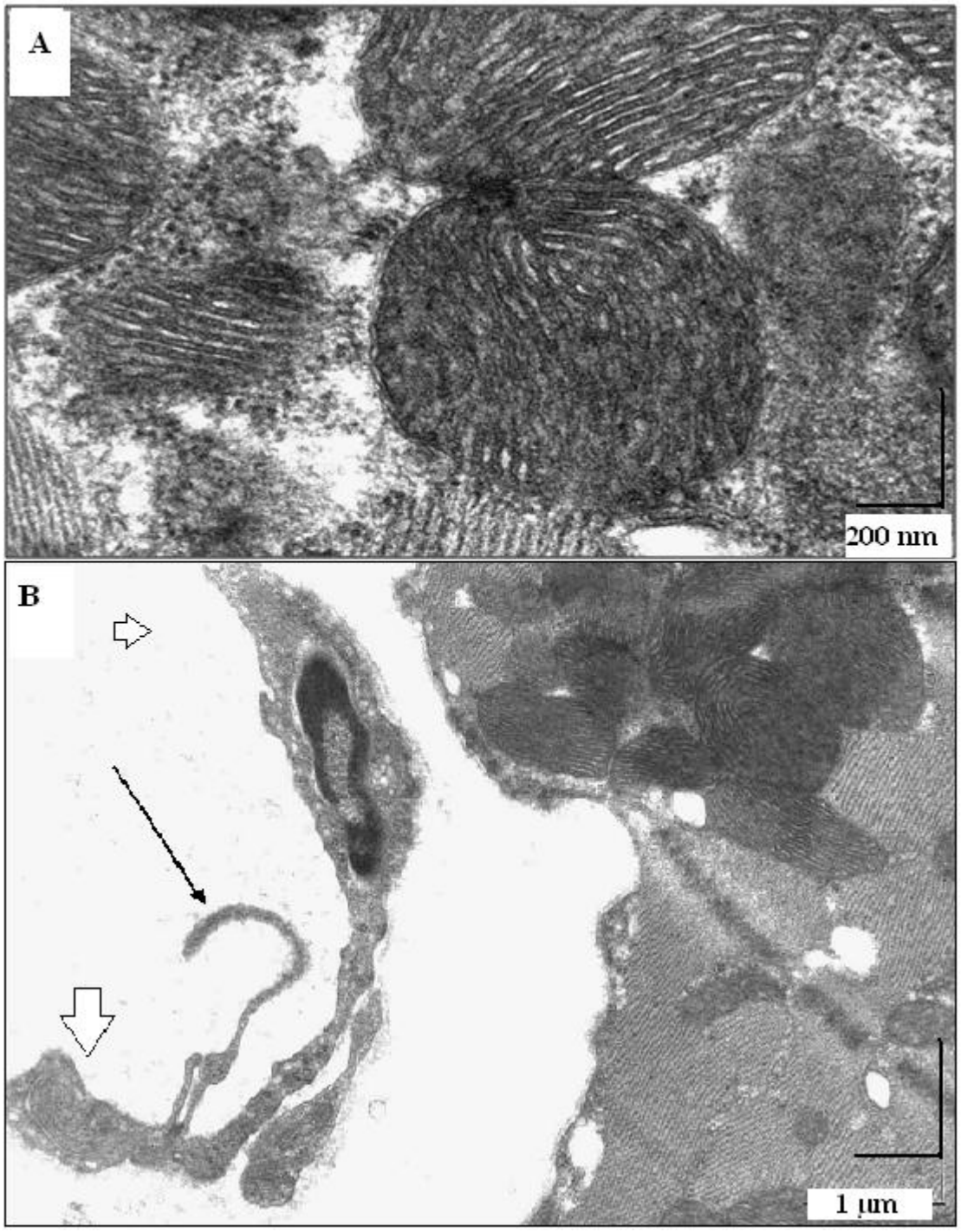


Figure 3.11: Electron photomicrograph of heart tissue in AGE- treated animals. (A) Normal shape mitochondria and increased glycogen deposition. (B) Increased transcytoplasmic vesicles (white arrows) in the capillary wall. Note also the intraluminal cytoplasmic extension at the junctional region (thin black arrow).

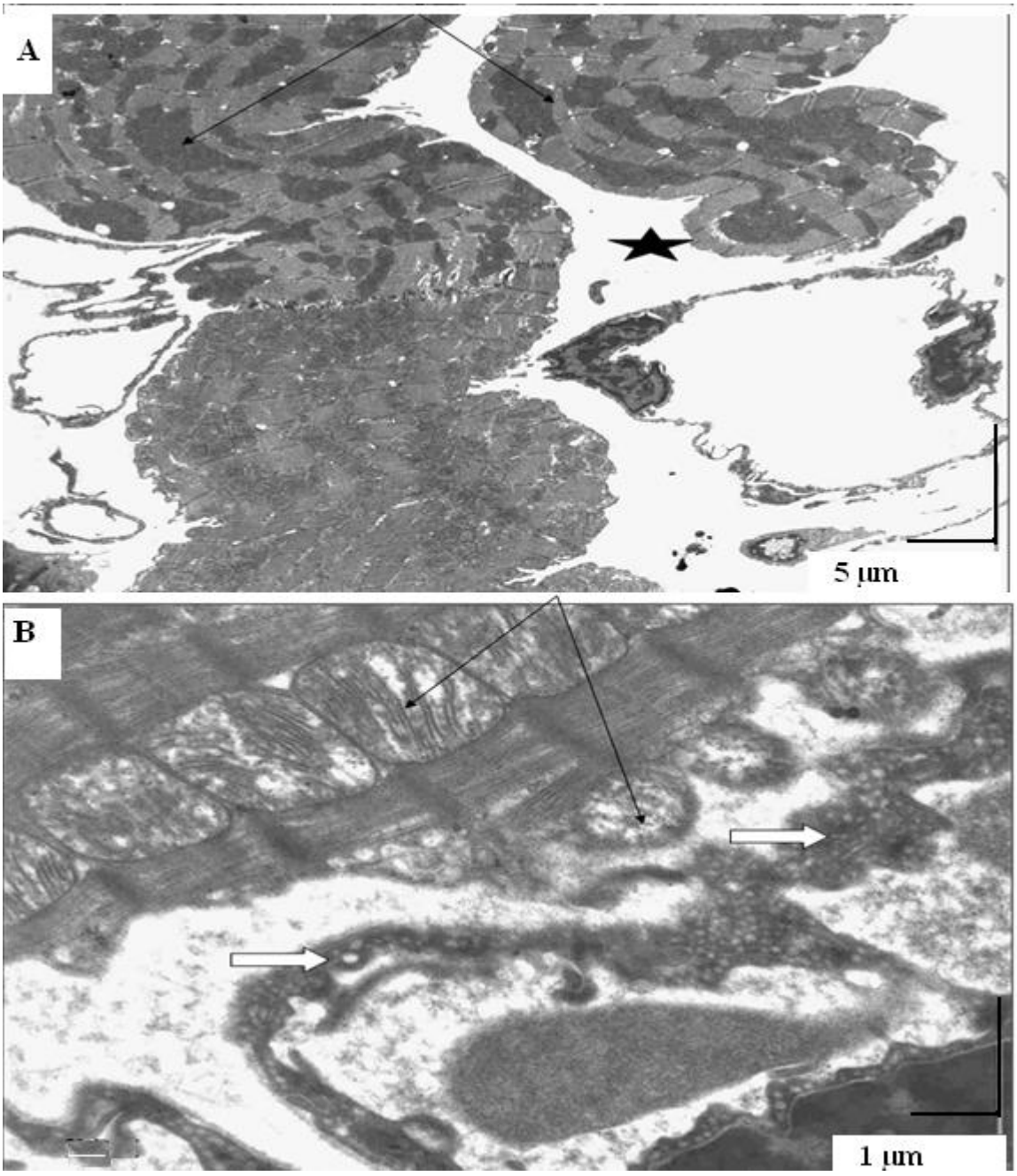


Figure 3.12: Electron photomicrograph of heart tissue in DOX- treated animals. (A) Irregular wavy fibres, separated by wide tissue spaces (star). The cytoplasm contains tiny vacuoles (arrows) and dense mitochondria. Blood capillaries showed an irregular wall. (B) Increased trancytoplasmic vesicles in the wall blood capillaries (white arrows). Mitochondria were enlarged showing less dense matrix (thin black arrow).

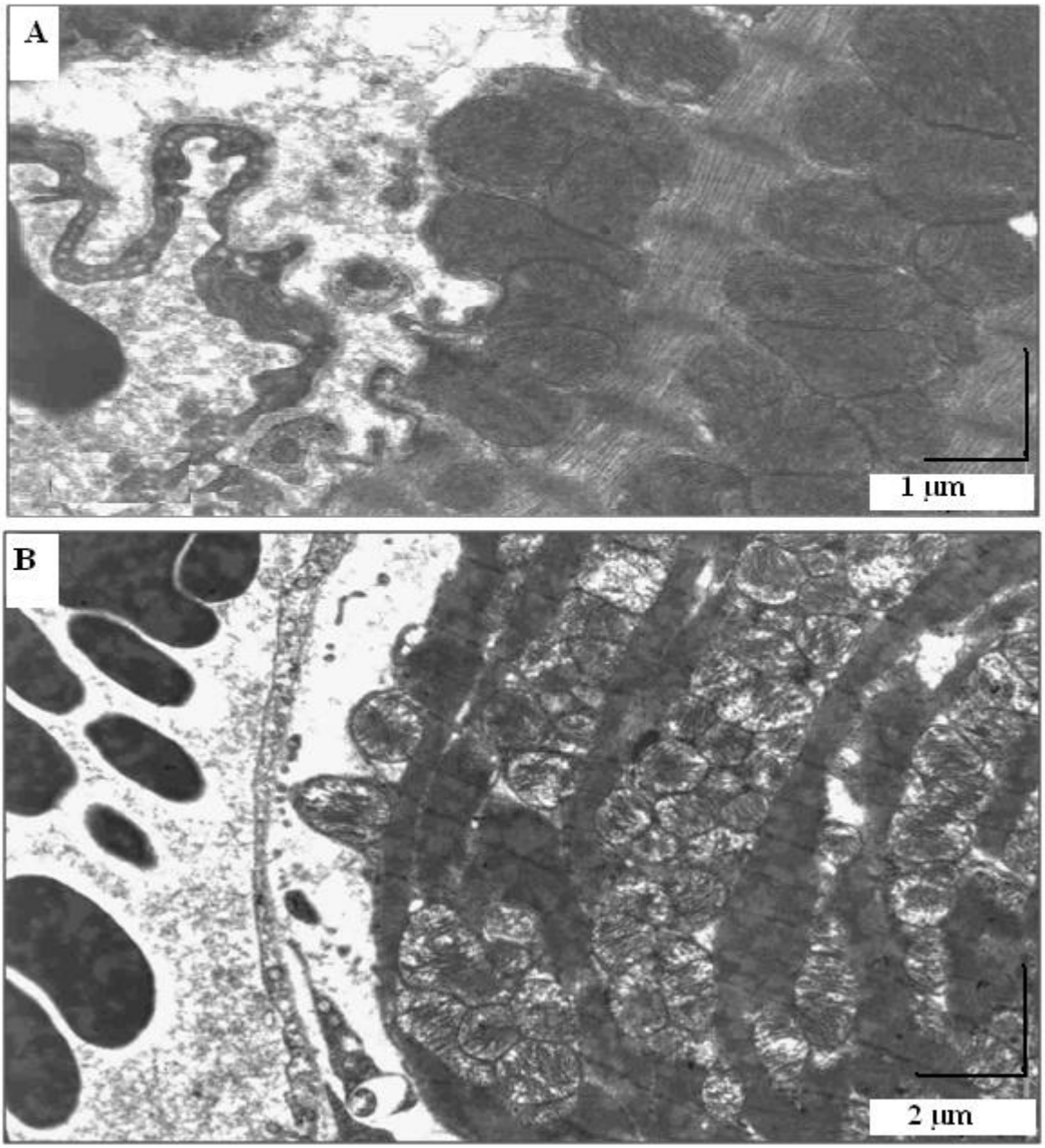


Figure 3.13: Electron photomicrograph of heart tissue in AGE + DOX- treated animals. (A) Transcytoplasmic vesicles were increased in capillary wall. (B) Vascular congestion and increase in blood vessels with AGE pre-treatment.

Animal Experiment Part 2

Survival of mice bearing EAC tumours

Table 3.1 and Figure 3.14 show the effects of AGE pre-treatment on the cytotoxic activity of DOX against the growth of EAC cells inoculated i.p. into Swiss albino mice. Control tumour-bearing mice showed a mean survival time (MST) of 17 days, whereas, administration of DOX (2 mg/kg) for 3 doses increased the MST to 50 days, with 30% long term survivors. Aged garlic extract pre-treatment with 2860 mg/kg p.o. once daily for six days, increased the MST of tumour bearing mice treated with DOX to 88 days with 70% long-term survivors.

Effects of aged garlic extract pre-treatment on doxorubicin uptake in EAC-cells

Table 3.2 shows the cellular level of DOX in EAC-cells following treatment with a single dose of DOX (15 mg/kg) and/or after daily administration of AGE 2860 mg/kg for 10 days. AGE pre-treatment significantly increased the cellular level of DOX 6 hours after treatment (144.85 ng/108 cells compared with 81.11 ng/108 cells for DOX alone).

Effects of aged garlic extract pre-treatment on doxorubicin tissue distribution

Figure 3.15 shows the calibration curve of DOX. Figures 3.16–3.19 demonstrate tissue distribution of DOX (15mg/kg, i.p.) alone and /or after AGE pre-treatment (2860 mg/kg,p.o.) daily for 10 days in serum heart, liver and kidney of female Swiss albino mice at 24, 48, 72 and 120 hours.

There was a significant increase in concentration of DOX in the serum of mice pre-treated with AGE (2860 mg/kg,p.o.) daily for 10 days at 24 hours ($p< 0.001$) as compared to mice treated with DOX (15 mg/kg) alone. The serum concentration of DOX in mice pre-treated

with AGE was significantly lower than in mice treated with DOX alone at 48 hours ($p < 0.05$) (Figure 3.16).

There was no significant change in concentration of DOX in the heart between the DOX-treated group and combined drugs treated group at all times (Figure 3.17). Moreover, there was a significant decrease in the concentration of DOX in the liver of mice pre-treated with AGE (2860 mg/kg, p.o.) daily for 10 days at 24 and 72 hours ($p<0.05$) when compared with mice treated with DOX (15 mg/kg) alone. The liver concentration of DOX in mice pre-treated with AGE was significantly higher than in mice treated with DOX alone after 48 hours ($p<0.05$) (Figure 3.18). There was a significant decline in the concentration of DOX in the kidneys of mice pre-treated with AGE (2860 mg/kg, p.o.) daily for 10 days at 24 and 48 hours ($p<0.05$) as compared to mice treated with DOX (15 mg/kg) alone (Figure 3.19).

Table 3.1: Effect of aged garlic extract pre-treatment on the antitumor activity of DOX in mice bearing EAC cells.

Groups	MST	LTS %
Control	17.4±0.8432	0
AGE	17.88±1.054	0
DOX	50±9.345*	30
AGE plus DOX	88±11.532*	70

MST (mean survival time) = average survival days of mice. LTS (long term survivors) are defined as the mice who survived to the end of experiment (90 days) without an apparent evidence of tumour cell growth.*Indicates significant change from control ($p<0.05$, one way ANOVA with LSD post test)

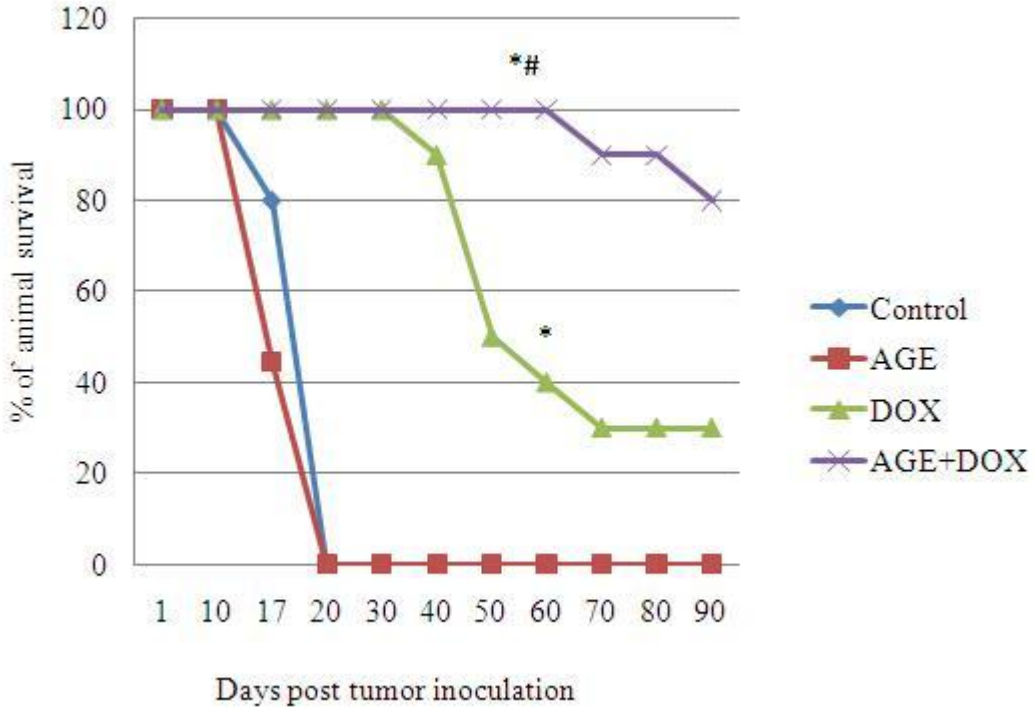


Figure 3.14: Effect of AGE pre-treatment on the anti-tumour activity of DOX in mice bearing EAC cells. Changes in % animal survival expressed as mean ± SEM. Each group consists of 10 animals.*p< 0.05, **p < 0.001 when compared with control, one way ANOVA with LSD post test).

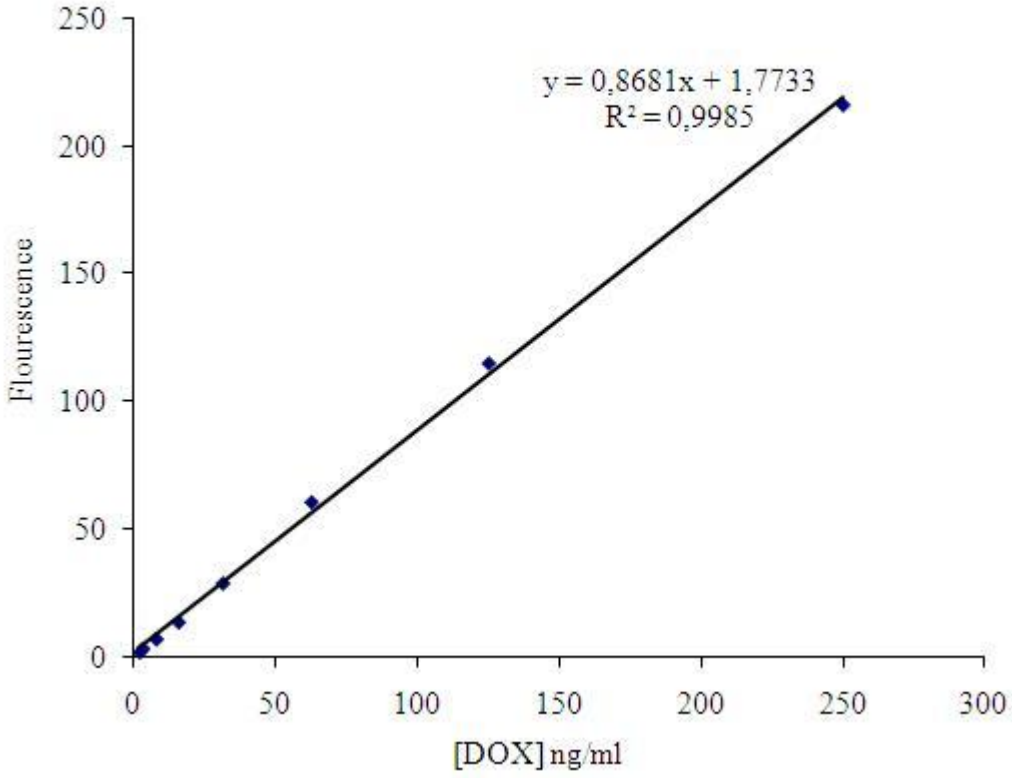


Figure 3.15: The calibration curve of DOX. This curve is typical of 3 different experiments.

Table 3.2: Tumour cells concentration of DOX (single dose of 15 mg/kg, i.p.) alone and /or with AGE pre-treatment (2860 mg/kg) in mice bearing EAC cells.

Groups / Time	DOX (ng/10X10^6 Cells)	DOX+AGE (ng/10X10^6 Cells)	p-value
6 hours	81.11±10.21	144.85±24.23	0.036*

The values are presented as mean ± SEM (n=6). * Significant difference at $p<0.05$, unpaired student t-test)

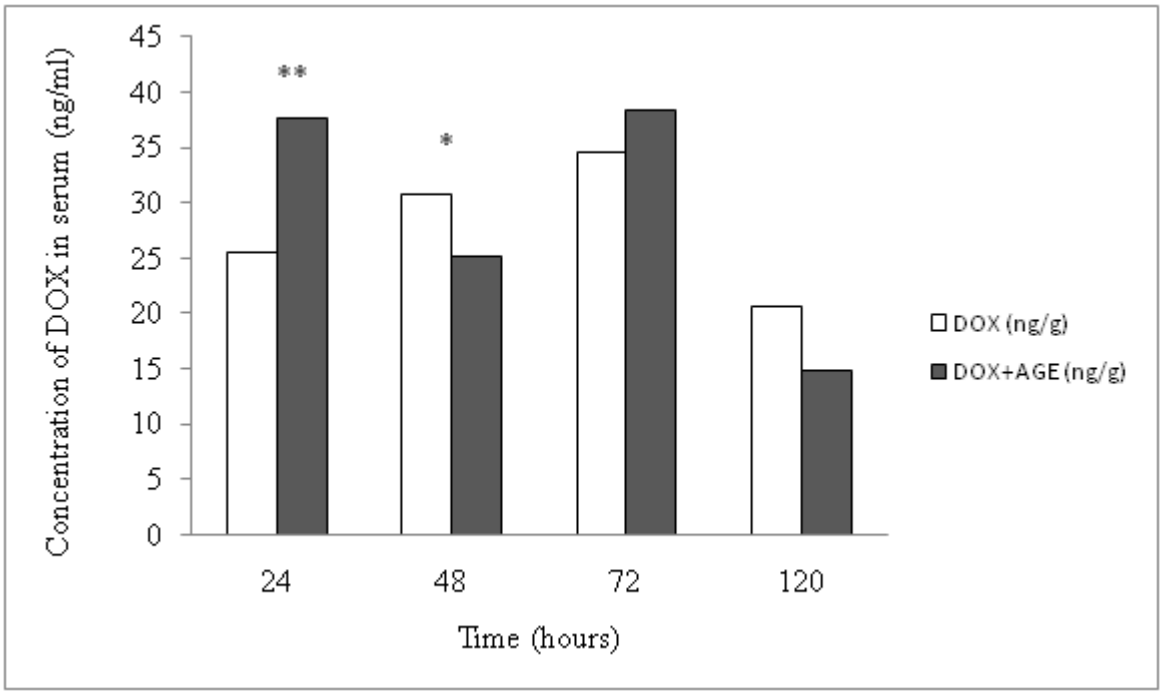


Figure 3.16: Serum concentration/time profiles of DOX. Concentration/time profiles of DOX (single dose of 15 mg/kg, i.p.) alone and/or with AGE pre-treatment (2860 mg/kg p.o.) daily for 10 days in serum of mice bearing EAC. The values are represented as mean ± SEM (n=6). * Significant difference at $p<0.05$, **$p < 0.001$, one way ANOVA with LSD post test.

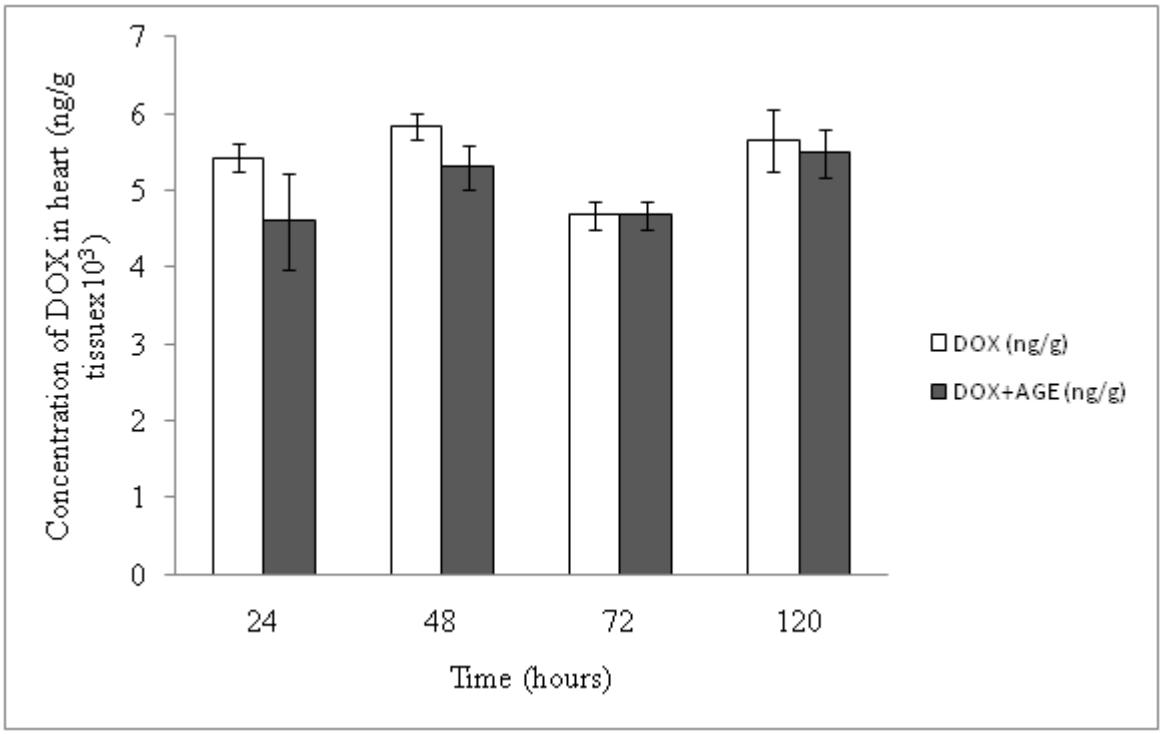


Figure 3.17: Heart concentration/time profiles of doxorubicin. Concentration/time profiles of DOX (single dose of 15 mg/kg i.p.) alone and/or with AGE pre-treatment (2860 mg/kg p.o.) daily for 10 days in heart of mice bearing EAC. The values are presented as mean ± SEM (n=6). No significant differences between DOX and DOX+AGE ($p>0.05$, unpaired student t-test).

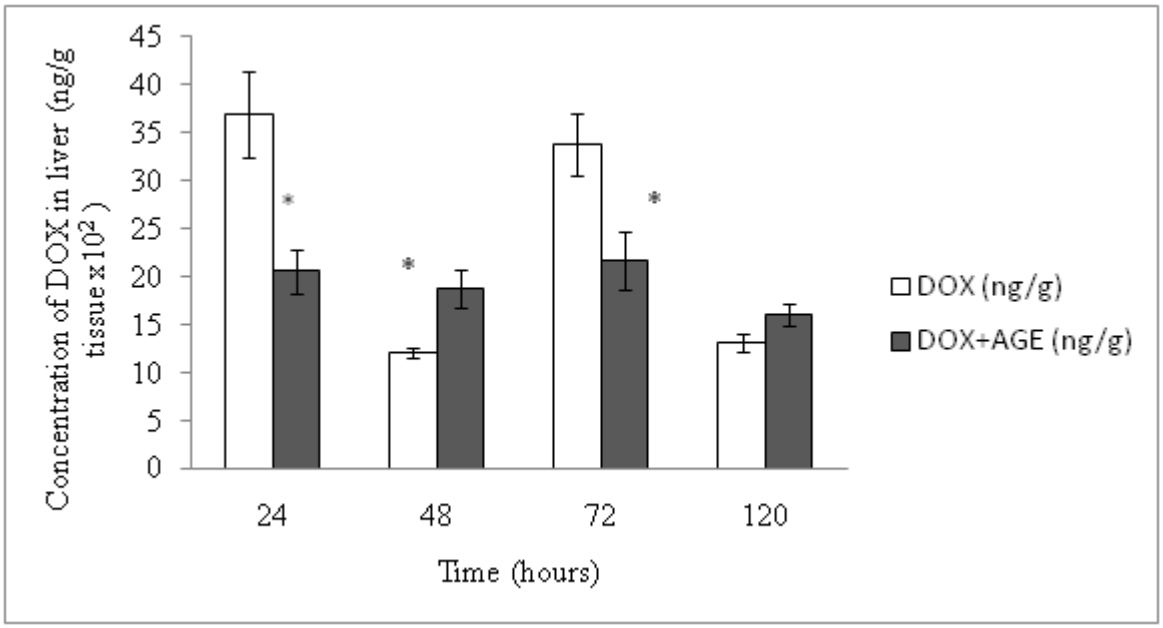


Figure 3.18: Liver concentration/time profiles of DOX. Concentration/time profiles of DOX (single dose of 15 mg/kg i.p.) alone and/or with AGE pre-treatment (2860 mg/kg p.o.) daily for 10 days in liver of mice bearing EAC. The values are presented as mean ± SEM (n=6). * Significant difference at $p<0.05$, unpaired student t-test.

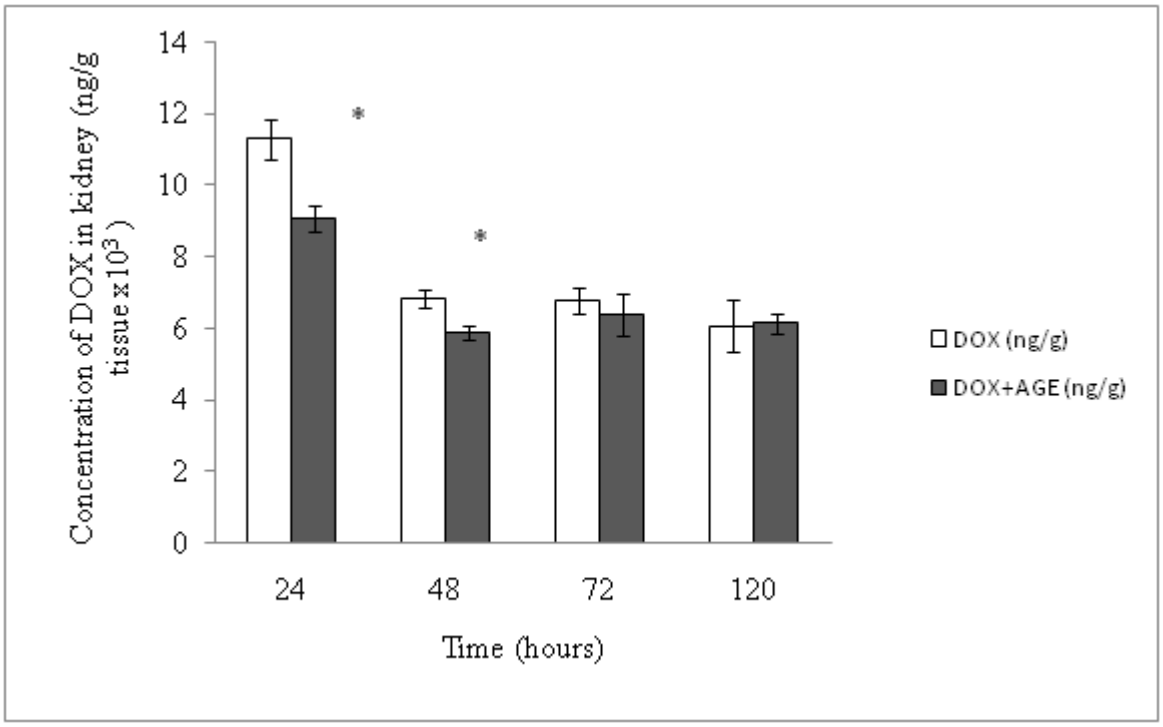


Figure 3.19: Kidney concentration/time profiles of DOX. Concentration/time profiles of DOX (single dose of 15 mg/kg i.p.) alone or with/without AGE pre-treatment (2860 mg/kg p.o.) daily for 10 days in kidney of mice bearing EAC. The values are presented as mean ± SEM (n=6). *Significant difference at $p<0.05$, unpaired student t-test.

3.4 Discussion

The ability of AGE to protect the heart against DOX-induced cardiotoxicity was tested in the current study. Aged garlic extract reduced the manifestation of DOX-induced cardiotoxic effects in rats. In the present study an animal model of acute cardiotoxicity was produced. The animal model used in this study is similar to previous reports (Kojima *et al.*, 1994; Kang *et al.*, 1996; Wu and Kang, 1998; Monnet and Orton, 1999; Nagi and Mansour, 2000; Al-Majed *et al.*, 2002; Liu *et al.*, 2002; Yagmurca *et al.*, 2003).

The dose of DOX used in this study to induce cardiotoxicity in rats is similar to that used in other similar studies investigating the effects of DOX on serum cardiac enzymes in rats. Koti *et al.* (2009) studied the effects of lipostat on DOX-induced cardiotoxicity in albino rats. They showed that doxorubicin at a dose of 15mg/kg for two weeks induced cardiotoxicity, which was confirmed by a significant increase in cardiac enzyme biomarkers (CPK and LDH). Ibrahim *et al.* (2010) also showed that DOX at a dose of 2.5 mg/kg/twice weekly/for three weeks in Wistar rats caused significant elevation in serum levels of LDH and CPK of 182.4% and 183.6%, respectively, when compared to the normal values. In another study conducted by Venkatesan (1998), the use of a single dose of DOX 30 mg/kg in male Wistar rats caused a significant increase in serum concentration of CPK and LDH compared with controls ($p<0.001$). The concentration of CPK and LDH increased by double when compared with control groups. Similarly, Mohamed *et al.* (2000) and Tatlidede *et al.* (2009) reported that serum CPK and LDH were increased in male Wistar rats following treatment with 20 mg/kg of DOX.

Studies investigating the effects of AGE on DOX-induced cardiotoxicity are limited. The ability of AGE to protect against DOX induced cardiotoxicity in rats was reported by

Demirkaya *et al.* (2009). Moreover, it was found that AGE at a dose of 600 mg/kg/day p.o. for six weeks administered with 3.750 mg/kg DOX i.p. once a week for 4 weeks (total cumulative dose of 15 mg/kg) in Wistar rats caused a significant decrease in DOX-induced cardiac injury as determined histopathologically. The protective effect of AGE was more obvious in the electron microscopic evaluation. Similar to the present study, Demirkaya *et al.* (2009) report severe mitochondrial swelling, the disappearance of cristae, and a loss of myofibrillary structure in the DOX-treated group, all of which were markedly decreased in the AGE+DOX group. Another study by Kojima *et al*. (1994) reports that AGE (WG-1, a preserved stock solution; Wakunaga Pharmaceutical) in conjunction with 1.5 mg/kg DOX administered i.p. three times per week for 40 days (total cumulative dose of 25 mg/kg) highlights significantly less lipid peroxidation as well as no significant pathological heart lesions in mice compared with those treated with DOX alone.

The results of DOX effects on MDA are in agreement with other studies in which DOX-induced increased plasma and heart lipid peroxidation products either throughout 24 hours following DOX administration, on the 3^{rd} 4^{th} days of DOX post-dosing injection or after cumulative treatment schedules (Luo *et al.*, 1997; Wu and Kang, 1998; Luo *et al.*, 1999; Antonio *et al.*, 2005; Lai *et al.*, 2010; Machado *et al.*, 2010; Patel *et al.*, 2010; Yalcin *et al.*, 2010). Treatment of male Wistar rats with a single dose of DOX 15 mg/kg, i.p. resulted in a significant increase in MDA 4 days following DOX administration ($p<0.05$ vs. control) (Elberry *et al.*, 2010). Öz *et al.* (2006) report that DOX single dose 45 mg/kg significantly increased plasma MDA in male Wistar rats after 24 hours of DOX administration ($p<0.05$).

Several studies suggest that DOX toxicity is related to oxidative stress and ROS generation (Bagchi *et al.*, 1995, Khan *et al.*, 2006, Ana Lucia Anjos *et al.*, 2007, Injac *et al.*, 2008). Zhou *et al.* (2001) found that cardiac myocytes isolated from rats following 6 weekly s.c.

injections of DOX (2 mg/kg) or an equivalent volume of saline showed a much higher rate of ROS formation compared to saline controls. This higher rate of ROS formation continued for 5 weeks following the last injection.

In the current study, AGE minimized DOX induced oxidative stress which was manifested as reduced serum TAS, and increased lipid peroxidation products in the plasma and hearts from DOX treated animals. The increase in MDA production caused by DOX was greatly reduced in AGE+DOX-treated rats. The increased oxidative stress caused by DOX caused peroxidation of membrane lipids measured as MDA (DOX vs. control; AGE+DOX vs. DOX) probably altering normal cell function, since polyunsaturated fatty acids are usually considered highly susceptible to ROS attack.

In the present study, AGE lowering effect on MDA production could be due to the presence of, SAC, the most abundant organosulfur compound in aged garlic extract. Previous studies have suggested that SAC act as a scavenger of superoxide radical and it also increases Cu/Zn SOD activity (Kim *et al.,* 2001). Aged garlic extract and its constituents have been reported to protect liver membranes from lipid peroxidation. It prevents both the formation of lipid peroxides and the physical damage they cause to membranes such as decrease of membrane fluidity or ability to exchange nutrients and waste across the membrane.

It has been reported that the addition of AGE to liver cells results in a significant reduction in MDA concentration (Horie *et al.*, 1989). Further studies by Horie *et al.* (1992) showed that the polysulphide fraction of AGE also significantly prevents lipid peroxidation of liver microsomes. It has been reported that AGE and S-allylcysteine (SAC) significantly prevent membrane damage, loss of cell viability, and lipid peroxidation in bovine pulmonary artery endothelial cells exposed to oxidized LDL (Durak *et al.*, 2004).

Aged garlic extract possesses strong antioxidant activity. A study found that the treatment of streptozotocin-induced diabetic rats with 500 mg/kg garlic daily caused recovery of antioxidant activity reaching levels in excess of those observed in normal rats (Drobiova *et al.*, 2009).

The present study demonstrated that AGE enhanced the cytotoxic activity of DOX against the growth of Ehrlich ascites carcinoma cells. There is no reported study concerning the effects of AGE on anti-tumour effects of DOX. Some studies have shown that garlic possesses anti-tumour activity (Omar and Al-Wabel, 2009; Seki *et al.*, 2000; Karasaki *et al.*, 2001; Kasuga *et al.*, 2001; Chang *et al.*, 2005). Garlic seems to target multiple pathways, including the inhibition of the mutagenesis, modulation of enzyme activities inhibition of DNA adduct, affecting the intrinsic pathway for apoptotic cell death and cell cycle machinery, all of which may lead to its anticancer activities (Sparnins *et al.*, 1986; Sparnins *et al.*, 1988; Zhang *et al.*, 1989; Lin *et al.*, 1994; Schaffer *et al.*, 1996; Hageman *et al.*, 1997). It has been further proposed that the anticancer effect is owing to the organosulphur compounds in the garlic (Knowles and Milner, 1998). Moreover, the inhibition of tumour cell proliferation by organosulphur compounds has been described in several studies using different cell cultures (Sundaram and Milner, 1993; Takeyama *et al.*, 1993; Sakamoto *et al.*, 1997; Seki *et al.*, 2000).

In the present study, AGE did not show anti-tumour activity by itself, but rather increased the anti-tumour activity of DOX against the growth of Ehrlich cells. Weisberger and Pensky (1958) have demonstrated *in vitro* and *in vivo* that thiosulfinate extracts of garlic inhibited the growth of malignant cells and also prevented the growth of sarcoma 180 ascites tumour. Recently, Hakimzadeh *et al.* (2009) studied the cytotoxic activity of garlic extract on Sk-

mel3 cell line of melanoma. They concluded that garlic is a potentially useful anti-tumour agent against melanoma.

Kasuga *et al.* (2001) conducted a study on ICR strain male mice (7 weeks old), which were inoculated in the hypoderm of the back with 10^6 cells of Sarcoma-180 in 100 µL of PBS. Twenty-four hours following the carcinoma cell inoculation, garlic preparations were administrated orally utilising a stomach tube at doses of 10ml/kg every other day for 3 weeks (n = 11 administrations). They conclude that AGE inhibited the growth of sarcoma-180 cells transplanted in mice and enhanced natural killer (NK) and killer cell activities. Previous studies have reported similar findings (Kyo *et al.*, 1998).

The current study showed that AGE alters the pharmacokinetics of DOX in mice. The study found that AGE increased the cytotoxic activity of DOX. This action may be owing to the suppression of P-glycoprotein associated energy-dependent efflux of DOX pump, leading to an increased intracellular drug concentration and increased cellular toxicity (Frézard *et al.*, 2001). In the present study, AGE produced higher serum level of DOX in aged garlic-pre-treated group and no increase in DOX concentration in cardiac tissue. Accordingly, it seems that AGE prevents the uptake of DOX in cardiac tissue but increases the tumour concentration of DOX which may be part of its cardioprotective effect

Previous reports showed that the decrease in the accumulation of DOX in tissues and its increase in plasma might be a function of pH gradient or p-glycoprotein over-expression. DOX is a weak base and its accumulation and toxicity in tissue has been investigated as a function of the extracellular pH; therefore, DOX levels in tissues increase by escalating the extracellular pH and, as a consequence, any drug which decreases the extracellular pH may decrease the accumulation of DOX in the tissues (Gerweck *et al.*, 1999).

In conclusion, the results of this study demonstrate that AGE protects against DOX-induced cardiotoxicity in rats. Moreover, AGE does not interfere with the cytotoxic activity of DOX but rather increases its activity against tumour cells in mice bearing EAC. The study is distinctive because there were only two studies so far that investigated the effects of AGE on some parameters involved in DOX-induced cardiotoxicity. The present study included the investigation of several factors involved in DOX-induced cardiotoxicity. These findings provide a window for reduction of the serious cardiac complication by natural products, such as AGE.

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Chapter 4 Effect of aged garlic extract against doxorubicin-induced cardiotoxicity in rat cardiac myocytes

4.1 Introduction

Doxorubicin-induced cardiotoxicity has been linked with increased oxidative stress which results in damage to macromolecules, membranes, DNA and enzymes involved in energy production, thereby leading to cellular damage, energy deficit and acceleration of cell death through apoptosis and necrosis (Singal *et al.*, 2000; Tokarska-Schlattner *et al.*, 2006). Doxorubicin has been reported to increase the levels of 8-isoprostane (Fujimura *et al.*, 2009). A marker of lipid peroxidation, 8-isoprostane, has been found to be the best index for determining oxidative injury through the utilisation of an oxidant stress rat model (Gross *et al.*, 2005; Morrow, 2005; Hwang and Kim, 2007).

In the heart, many previous investigations have designated apoptosis of cardiac myocytes as the most direct cause of DOX cardiotoxicity (Kalyanaraman *et al.*, 2002; Bernuzzi *et al.*, 2009; Gilleron *et al.*, 2009; Chao *et al.*, 2011; Chen *et al.*, 2011). The process of apoptosis is ultimately characterised by chromatin condensation and DNA fragmentation. This highly regulated mechanism is organised by cysteinyl-aspartate-cleaving proteases known as caspases. These enzymes cleave numerous and various substrates leading to cell disassembly (Kroemer *et al.*, 2007). Moreover, it is recognised that caspase-3 and p53 are two of the apoptotic mediators in the main apoptotic signalling pathways. Protein levels of p53 and caspase-3 activity tend to increase with DOX treatment (Hong *et al.*, 2010; Lai *et al.*, 2010).

Aged garlic extract is a potent antioxidant with established cardioprotective effects (Jacob *et al.*, 1993; Rahman and Billington, 2000; Thabrew *et al.*, 2000; Mukherjee *et al.*, 2003). Its antioxidant activity is ascribed largely to a key constituent called S-allylcysteine (SAC), which is a potent antioxidant and free radical scavenger (Imai *et al.*, 1994; Pérez-Severiano *et al.*, 2004; Medina-Campos *et al.*, 2007). Previous studies show that both AGE and SAC are effective cardioprotectants (Kojima *et al.*, 1994; Mukherjee *et al.*, 2003; Chuah *et al.*, 2007; Padmanabhan and Stanely Mainzen Prince, 2007). There is lack of information concerning the use of AGE *in vitro* for the attenuation of DOX-induced cardiotoxicity; accordingly, there is a need for this particular study.

This study investigates the protective effects of AGE against DOX-induced cardiotoxicity in rat cardiac myocytes. The objectives were:

To investigate the effect of AGE on cardiac myocyte growth using either the Coulter counter or Cell Titer 96® AQueous one solution cell proliferation assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS kit).

To determine the concentrations of 8-isoprostane in rat cardiac myocyte culture medium.

To determine the concentrations of active caspase-3 and the activity of active and total p53 in rat cardiac myocytes.

To detect apoptosis using propidium iodide (PI) /4,6-diamidino-2-phenylindole (DAPI) staining in rat cardiac myocytes.

4.2 Materials and Methods

Effect of different concentrations of doxorubicin, aged garlic extract or both on cell growth in cultured rat cardiac myocytes

Rat cardiac myocytes were cultured in cardiac myocyte complete medium, as described in Section 2.13 Cells were incubated with different concentrations of AGE for 8 hours, subsequently followed by incubation with DOX for 24 hours. Following incubation, cells were collected by centrifugation (1000g for 5 minutes), and biochemical parameters were measured as described in Chapter 2. The effect of DOX, AGE or both on cardiac myocyte growth was investigated by measuring the viability of the cells using the Coulter counter and Cell Titer 96® AQueous one solution cell proliferation assay [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] (MTS kit) as described in Section 2.14 and Section 2.15.

The following parameters were measured, as described in Chapter 2. The concentrations of 8-isoprostane in cardiac myocyte culture medium were measured by the method described in Section 2.8. The concentrations of active caspase-3 in rat cardiac myocytes were measured by the method described in Section 2.9. The activity of active and total p53 in rat cardiac myocytes was measured by the methods described in Section 2.10 and Section 2.11. Apoptosis in cardiac myocytes was detected using PI/DAPI staining according to the method described in section 2.16.

4.3 Results

The effect of doxorubicin on rat cardiac myocyte growth using the Coulter counter

The effect of different DOX concentrations on cardiac myocyte growth was studied using the Coulter counter method. Exposure of cardiac myocyteto 0.5, 1, 5, 10 and 20 µM DOX reduced their growth by 31.33%, 40.7%, 54.82%, 64.46% and 69.26%, respectively. As shown in Figure 4.1, the addition of DOX displayed both potent and dose-dependent inhibition of cardiac myocyte growth at the tested concentrations. Doxorubicin significantly inhibited cardiac myocyte growth at concentrations between 1 and 20 µM ($p < 0.001$).

The effect of aged garlic extract on rat cardiac myocyte growth using the Coulter counter

Figure 4.2 shows the effects of 1-50 µg/ml AGE on cardiac myocyte growth. The growth of cardiac myocytes treated with 1, 10, and 50 µg/ml AGE was decreased non-significantly by 28.70%, 23.73%, and 19.45%, respectively when compared with control. Meanwhile, at a concentration of 0.1, 0.5, 1, 5, and 10 mg, AGE resulted in a significant inhibition of cardiac myocyte growth. As shown in Figure 4.3, cardiac myocyte growth was significantly reduced in the presence of AGE at concentrations between 0.1 and 0.5 mg/ml compared with the control ($p<0.05$). Moreover, the addition of AGE at a concentration of 1–5 mg/ml displayed a highly significant inhibition (17%, 34.35%, and 51.88%) respectively on cardiac myocyte growth compared with the control (Figure 4.4, $p<0.001$).

The effect of doxorubicin or aged garlic extract or both on rat cardiac myocyte growth using the MTS assay

There was no significant difference in the cardiac myocyte growth treated with 1 µM of DOX or 1 and 10 µg of AGE (Figure 4.5). Meanwhile, 5 µM DOX produced a significant reduction of 43.64% in the growth of cardiac myocytes compared with the control. Moreover, cells pre-treated with 1, and 10 µg of AGE showed a decrease in the growth of 53.51% and 44% respectively, when compared with control cells (Figure 4.6; $p<0.05$). The exposure of cardiac myocytes to 10 µM of DOX resulted in 74.34% inhibition of cell growth compared with the control. Similarly, the pre-incubation of cells with AGE at a concentration of 1, and 10 µg caused significant reduction of 74.63% and 69.56% respectively (Figure 4.7; $p<0.05$). However, notably, there was no significant difference between DOX-treated cells and AGE+DOX-treated cells.

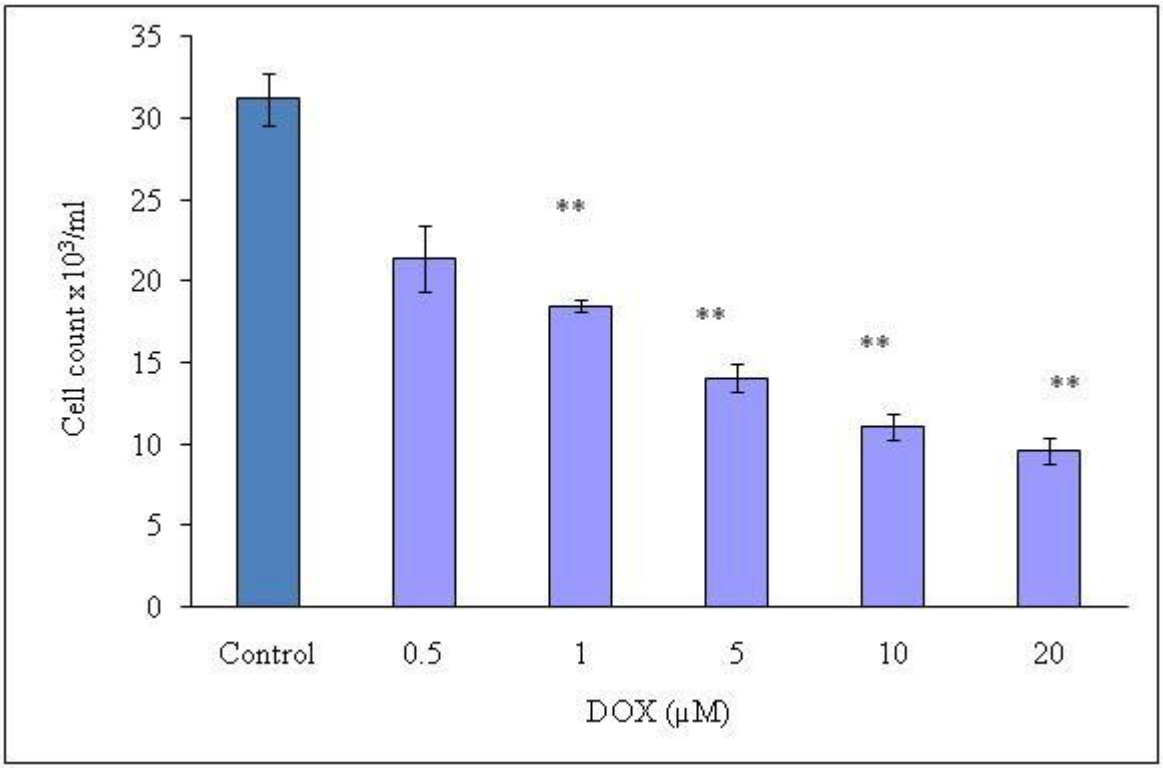


Figure 4.1: The effect of different concentrations of DOX on cardiac myocyte growth using the Coulter counter method. Cardiac myocytes (2 x 10^4 cells/ml) were incubated with different concentrations of 0.5 – 20 μM DOX for 24 hours and the number of cells counted using a Coulter counter. Results were expressed as mean ± SEM (n = 3). ** Significantly different from control ($p < 0.001$, one way ANOVA with LSD post test)

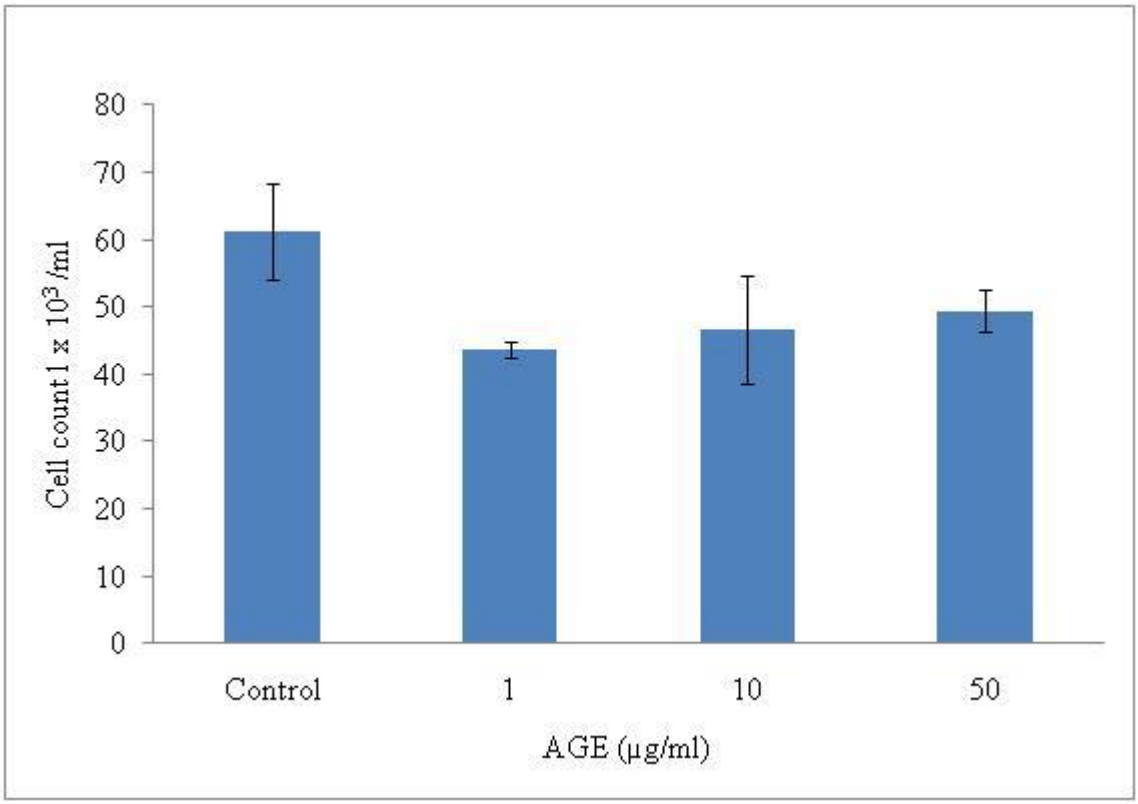


Figure 4.2: The effects of of AGE (1-50 µg/ml) on cardiac myocyte growth using the Coulter counter method. Cardiac myocytes (2 x 10^4 cells/ml) incubated with 1 – 50 µg/ml AGE for 24 hours and the number of cells were counted using a Coulter counter. Results were expressed as mean ± SEM (n = 3). No significant differences between groups ($p>0.05$, one way ANOVA).

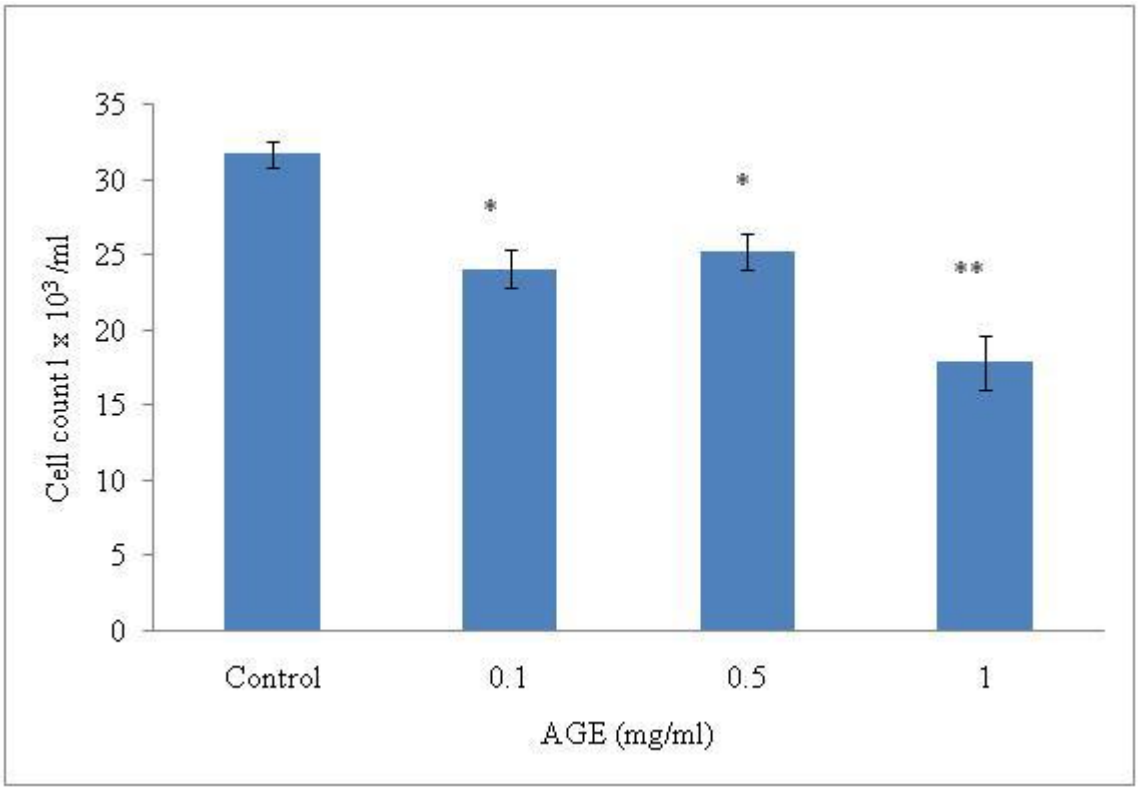


Figure 4.3: The effects of AGE (0.1-1mg/ml) on cardiac myocyte growth using the Coulter counter method. Cardiac myocytes (2 x 10^4 cells/ml) incubated with 0.1 –1mg/ml AGE for 24 hours and the number of cells were counted using a Coulter counter. Results were expressed as mean ± SEM (n = 3). *Significantly different from control ($p< 0.05$), ** Significantly different from control ($p< 0.001$, one way ANOVA with LSD post test).

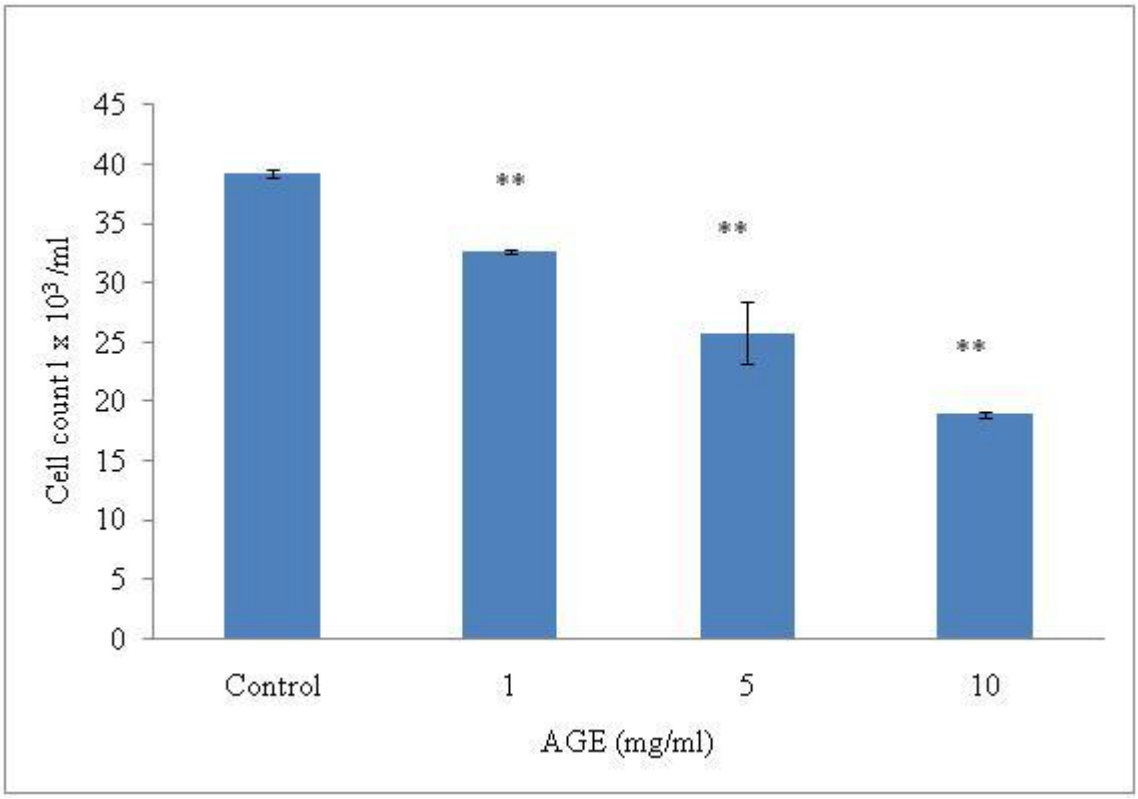


Figure 4.4: The effects of 1-10 mg/ml AGE on cardiac myocyte growth using the Coulter counter method. Cardiac myocytes (2 x 10^4 cells/ml) were incubated with 1 –10 mg/ml AGE for 24 hours and the number of cells were counted using a Coulter counter. Results were expressed as mean ± SEM (n = 3). ** Significantly different from control ($p< 0.001$, one way ANOVA with LSD post test).

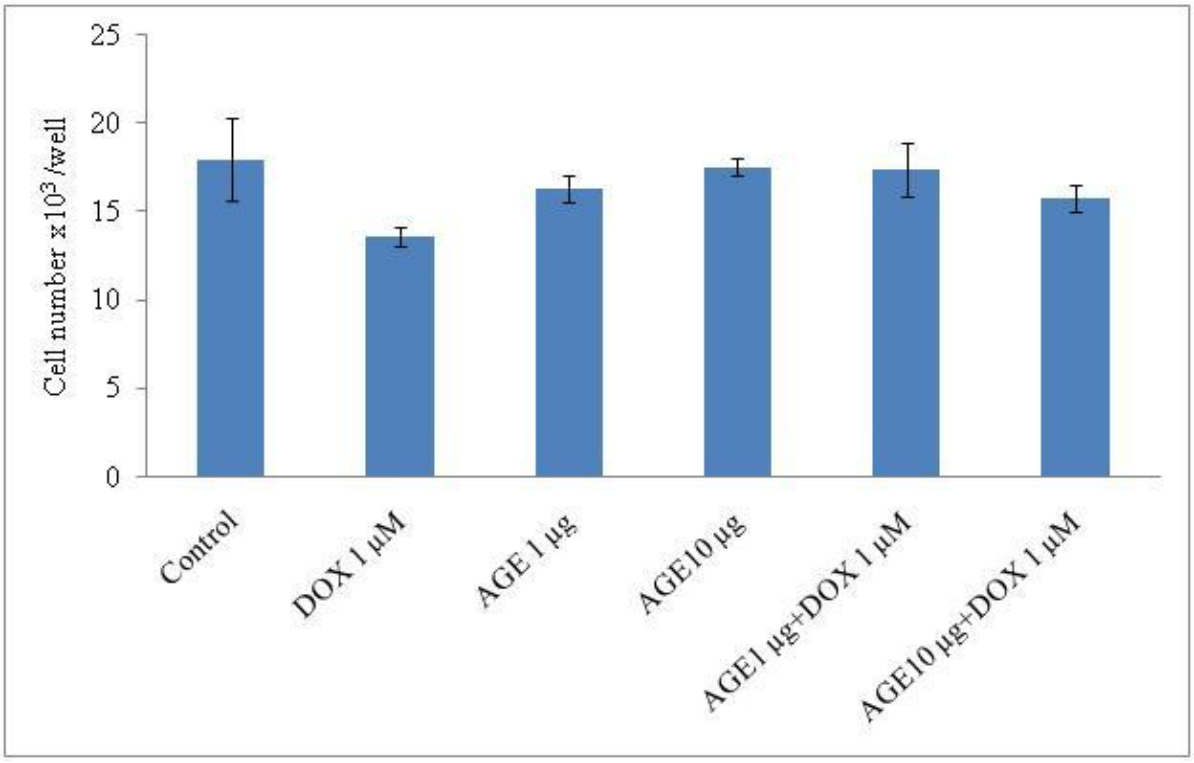


Figure 4.5: The effect of DOX 1µM on cardiac myocyte growth using the MTS assay. Cardiac myocytes (5 x 10^3 cells/well) were seeded in 96-well plates. After 8 hours treatment without (control) or with 1, and 10 µg AGE, cardiac myocytes were incubated with 1 µM DOX for 24 hours. The results were then presented as mean ± SEM (n=3). No significant differences between groups ($p>0.05$, one way ANOVA).

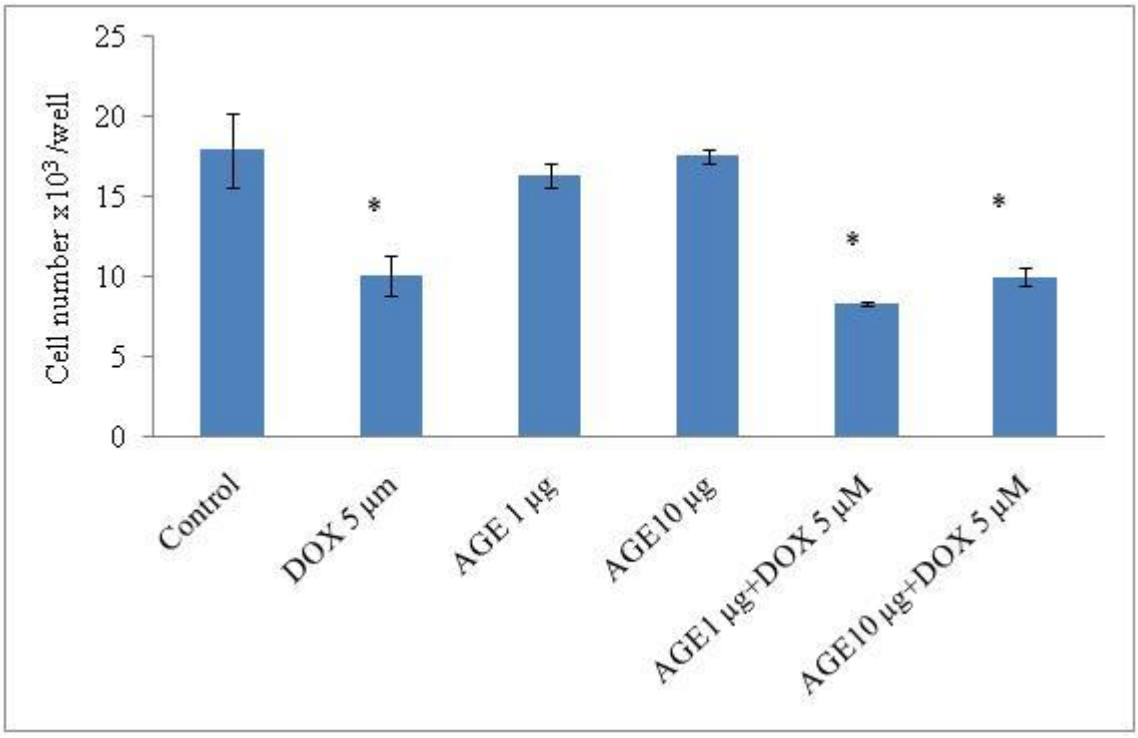


Figure 4.6: The effect of 5µM DOX on cardiac myocyte growth using the MTS assay. Cardiac myocytes (5 x 10^3 cells/well) were seeded in 96-well plates. After 8 hours treatment without (control) or with 1, and 10 µg AGE, cardiac myocytes were incubated with 5 µM DOX for 24 hours. The results were presented as mean ± SEM (n=3). * Significantly different from control ($p < 0.05$, one way ANOVA with LSD post test).

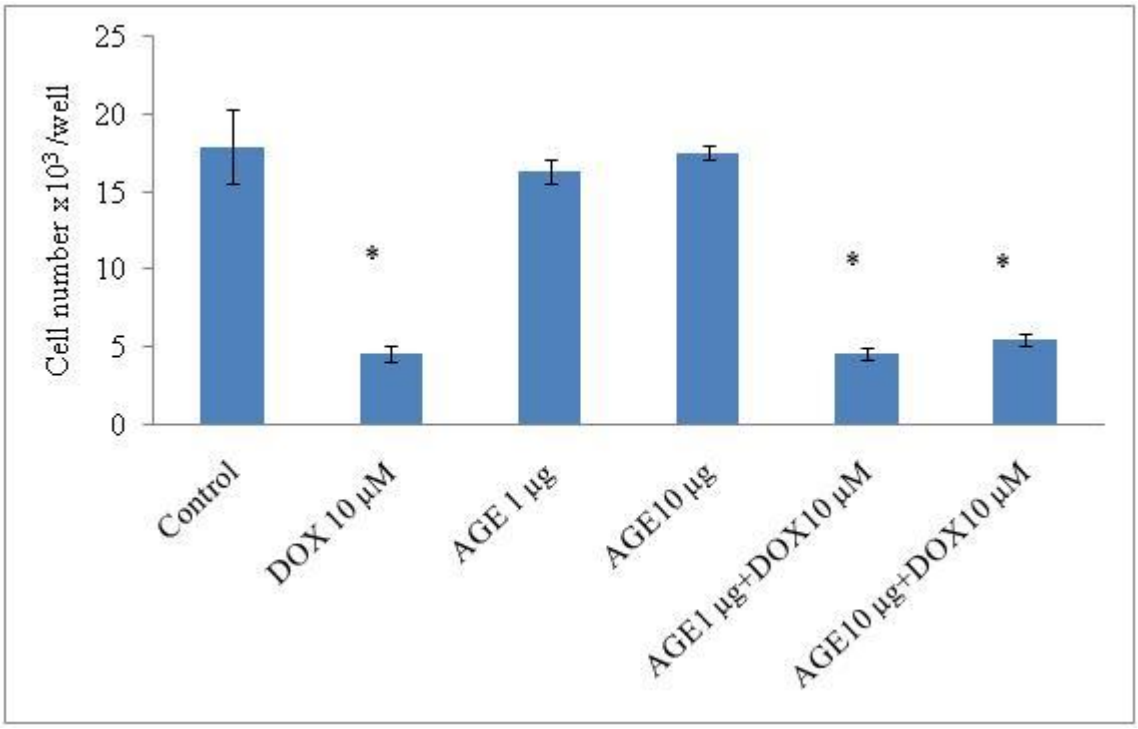


Figure 4.7: The effect of 10 µM DOX on cardiac myocyte growth using the MTS assay. Cardiac myocytes (5 x 10^3 cells/well) were seeded in 96-well plates. After 8 hours' treatment without (control) or with 1, and 10 µg AGE, cardiac myocytes were incubated with 10 µM DOX for 24 hours. The values are presented as mean ± SEM (n=3). *Significantly different from control ($p < 0.05$, one way ANOVA with LSD post test).

The levels of 8-isoprostane in rat cardiac myocyte culture medium

The effect of AGE on DOX-induced oxidative stress was tested using 8-isoprostane assay in culture medium. The amount of 8-isoprostane tracer which is able to bind to the rabbit antiserum is inversely proportional to the concentration of 8-isoprostane in the well. Doxorubicin (10 µM) was found to increase 8-isoprostane in cardiac myocyte culture medium significantly by 37.46% ($p<0.05$, Figure 4.8). The pre-incubation of cardiac myocytes with 1000 µg/ml AGE resulted in a significant decrease in 8-isoprostane levels by 8% compared with the control ($p<0.05$), and a significant decrease of 72.59% compared with the DOX-treated cells ($p<0.001$).

The concentration of active caspase-3 in rat cardiac myocytes

Figure 4.9 shows the effect of 10 µM DOX on the concentration of active caspase-3 in cardiac myocytes in the presence and absence of AGE 10, 100, and 1000 µg/ml. Caspase-3 activity in DOX-treated cardiac myocytes significantly increased by 17.01% when compared with control cells ($p<0.05$). Aged garlic extract at a concentration of 100 and 1000 µg/ml significantly inhibited DOX-induced caspase-3 activation by 48.31% and 31.13 % respectively, compared with the control cells. Similar reduction in the concentration of active caspase-3 was observed with the pre-incubation of cells with AGE 100 and 1000 µg/ml of 55.85% and 41.17% respectively, compared with DOX-treated cells ($p<0.05$). Active caspase-3 concentration in rat cardiac myocytes exposed to 10, 100, and 1000 µg/ml of AGE were decreased non significantly by 9.03%, 0.55%, and 12.61% respectively, compared with control cells. Pre-incubation of cells with 10 µg/ml AGE resulted in a non-significant reduction in active caspase-3 concentration by 7.14% compared to control and 20.67% compared to DOX-treated cells. Staurosporine (0.25 µM) was used as a positive

control and showed a significant increase in active caspase-3 activity by 23.61% compared with the control.

The activity of active and total p53 in rat cardiac myocytes

A significant increase in active p53 (approximately 125.56% over control; $p<0.05$) was observed after treatment of cardiac myocytes with 10 µM DOX (Figure 4.10). The treatment of cells with 10, 100, and 1000 µg of AGE revealed a non-significant increase in active p53 of 43.51%, 39.88%, and 7.41% respectively, compared with the control.

Pre-incubation of cells with AGE 1000 µg/ml significantly reduced DOX-induced active p53 production by 94.89%, compared with DOX-treated cells ($p<0.05$). Pre-incubation of cells with 10 and 100, µg of AGE caused a non-significant reduction in active p53 by 26.57% and 78.58%, and respectively compared with DOX-treated cells. Staurosporine (0.25 µM), the positive control, displayed a significant increase in active p53 activity by 167.11% compared with the control.

On the other hand, there was no noticeable change in the activity of total p53 in rat cardiac myocytes treated with the different conditions (Figure 4.11).

Detection of apoptosis in rat cardiac myocytes using propidium iodide / 4,6-diamidino-2-phenylindole staining

Apoptosis was assessed using PI /DAPI staining. The apoptotic and non-apoptotic nuclei were visualized using fluorescence microscopy (Figure 4.12). Cardiac myocytes treated with 10 µM DOX for 24 hours showed chromatin condensation and nuclear fragmentation, which is well known as typical apoptosis (Figure 4.12 C).

Pre-treatment of cardiac myocytes with 100 µg AGE reduced significantly the percentage of apoptotic cells from 826.19 ± 254.90% (DOX-treated cells) to 81.90 ± 8.87% (AGE+DOX-treated cells) ($P<0.05$; Figure 4.13). Apoptotic myocytes were defined as PI positive and DAPI negative. Propidium iodide positive cells appear red when DAPI is used to counter stain all nuclei. Non-apoptotic nuclei remained blue.

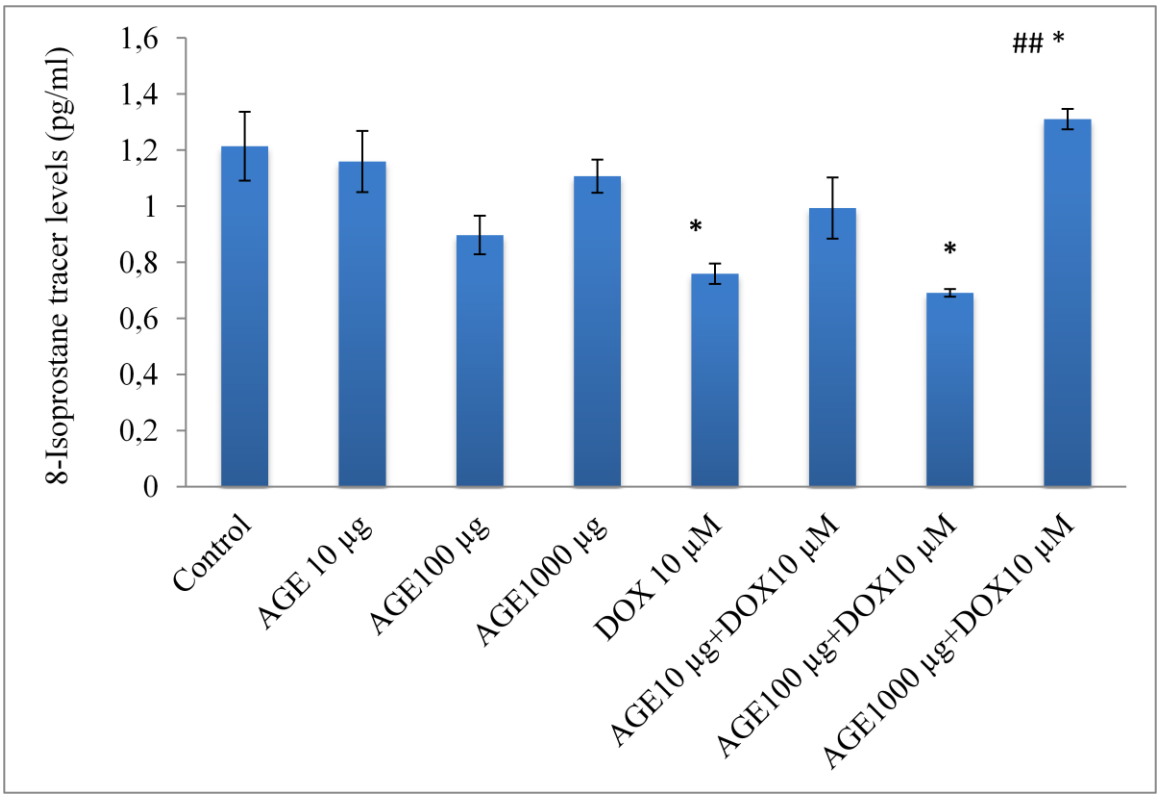


Figure 4.8: The effect of AGE on 8-isoprostane tracer levels in cardiac myocytes culture medium. After 8 hours' treatment without (control) or with 10, 100, and 1000 µg AGE, cardiac myocytes were incubated with 10 µM DOX for 24 hours. The values are presented as mean ± SEM (n=3). * Significantly different from control ($p< 0.05$). ## Significantly different from DOX ($p<0.001$, one way ANOVA with LSD post test).

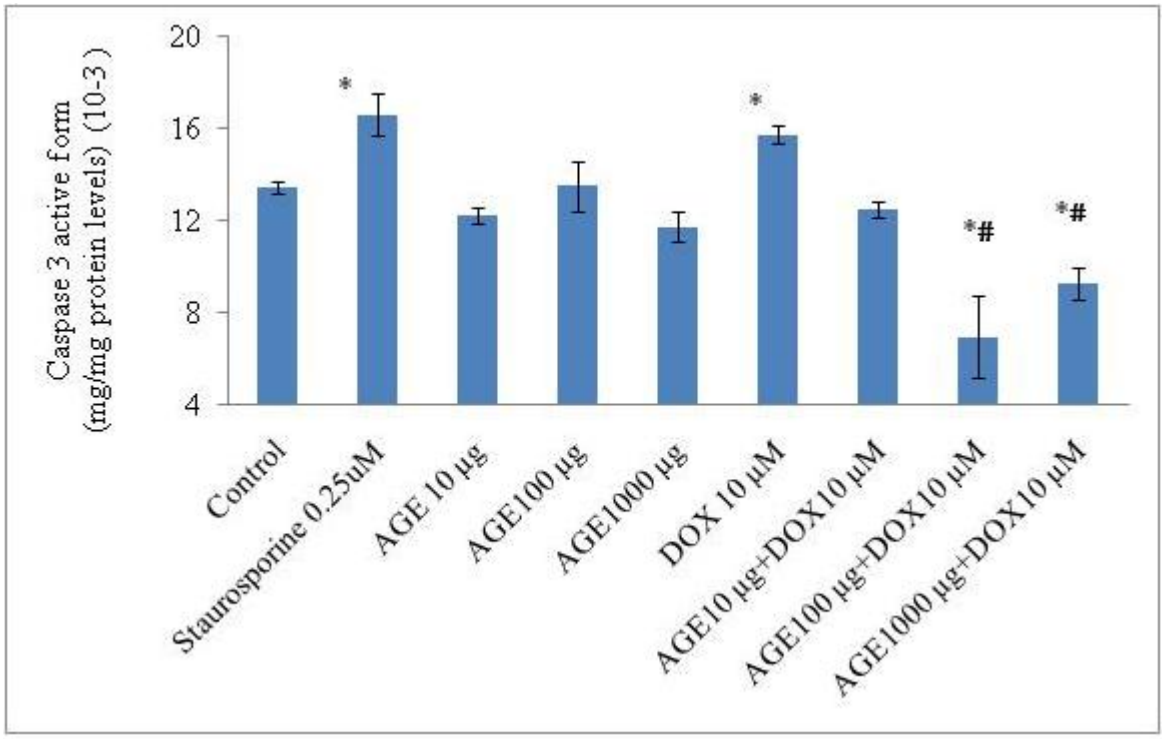


Figure 4.9: The effect of AGE on the concentrations of active caspase-3 in cardiac myocytes. After 8 hours' treatment without (control) or with 10, 100, and 1000 µg AGE, cardiac myocytes were incubated with 10 µM DOX for 24 hours. Staurosporine (0.25 µM) was used as a positive control. The values are presented as mean ± SEM (n=3). * Significantly different from control ($p < 0.05$). # Significantly different from DOX ($p<0.05$, one way ANOVA with LSD post test).

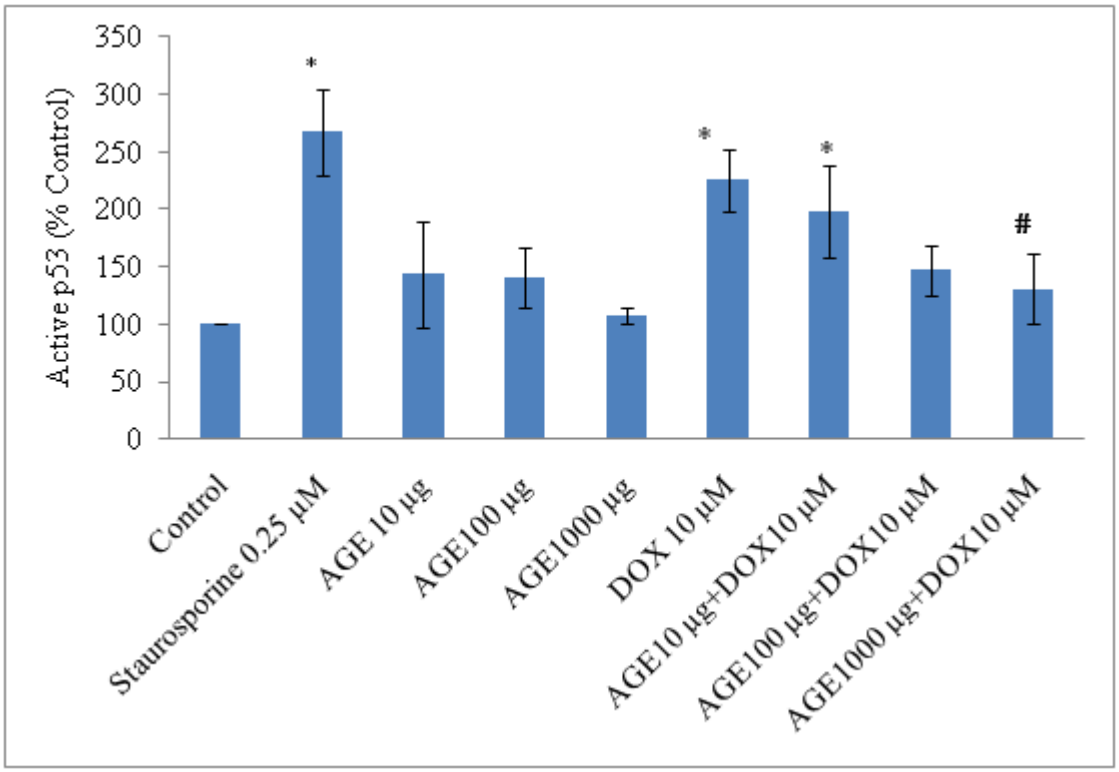


Figure 4.10: The effect of AGE on the activity of active p53 in cardiac myocytes. After 8 hours' treatment without (control) or with 10, 100, and 1000 µg AGE, cardiac myocytes were incubated with 10 µM DOX for 24 hours. Staurosporine (0.25 µM) was used as a positive control. The values are presented as mean ± SEM (n=3). * Significantly different from control ($p< 0.05$). # Significantly different from DOX ($p<0.05$, one way ANOVA with LSD post test).

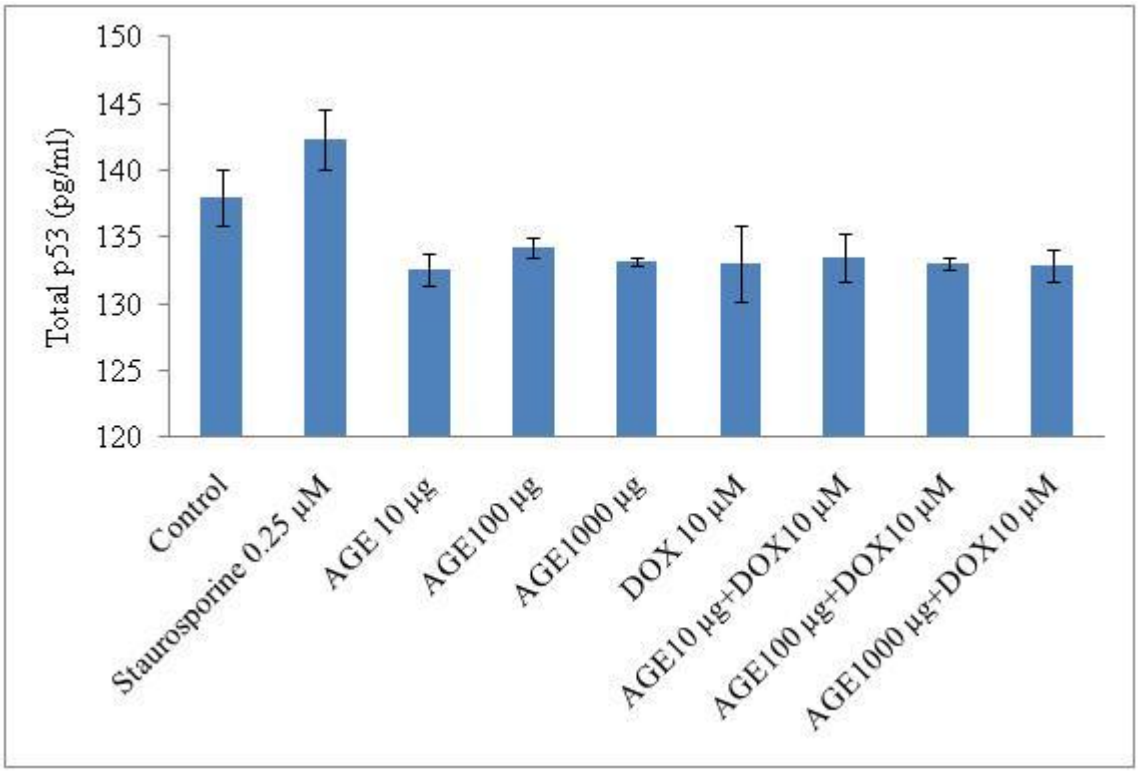


Figure 4.11: The effect of AGE on the activity of total p53 in cardiac myocytes. After 8 hours' treatment without (control) or with 10, 100, and 1000 µg AGE, cardiac myocytes were incubated with 10 µM DOX for 24 hours. Staurosporine (0.25 µM) was used as a positive control. The values are presented as mean ± SEM (n=3). No significant differences between groups ($p>0.05$, one way ANOVA).

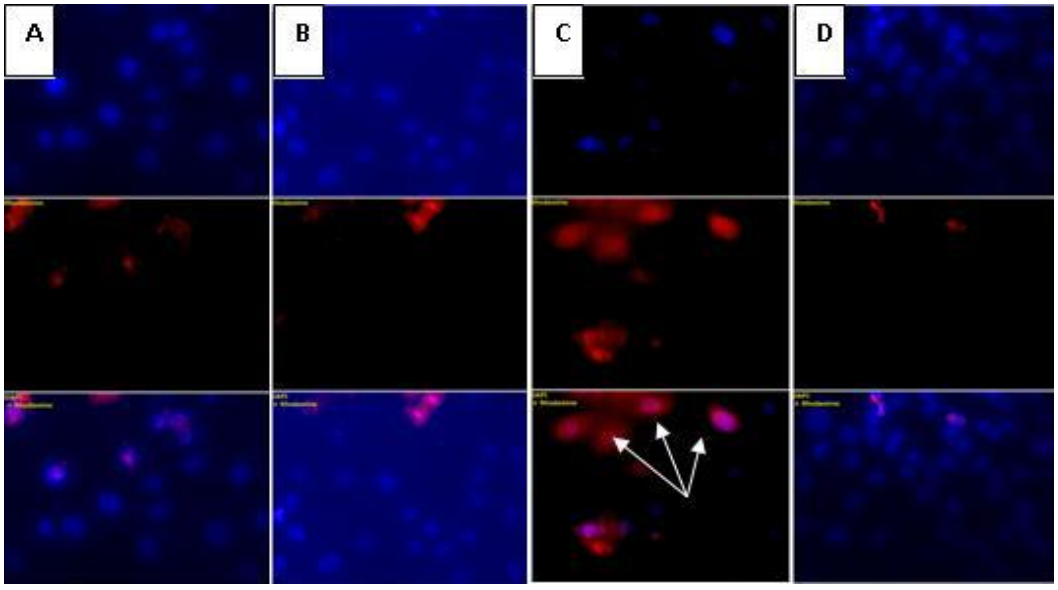


Figure 4.12: The effect of 100µg AGE on the morphology of cardiac myocyte treated with 10µM DOX. After 8 hours' treatment without (control) or with AGE (100 µg), cardiac myocytes were incubated with 10 µM DOX for 24 hours. Cells were fixed and stained with propidium iodide, and visualized under a fluorescence microscope. (A) Control, (B) 100 µg AGE, (C) 10 µM DOX, (D) 100 µg AGE + 10 µM DOX. Cardiac myocyte were stained by propidium iodide (P.I) method and DAPI as a counter stain. P.I positive cells appear red when DAPI is used to counter stain all nuclei. Moreover, non-apoptotic nuclei remained blue. The arrow indicates a positively stained apoptotic cell. (400x)

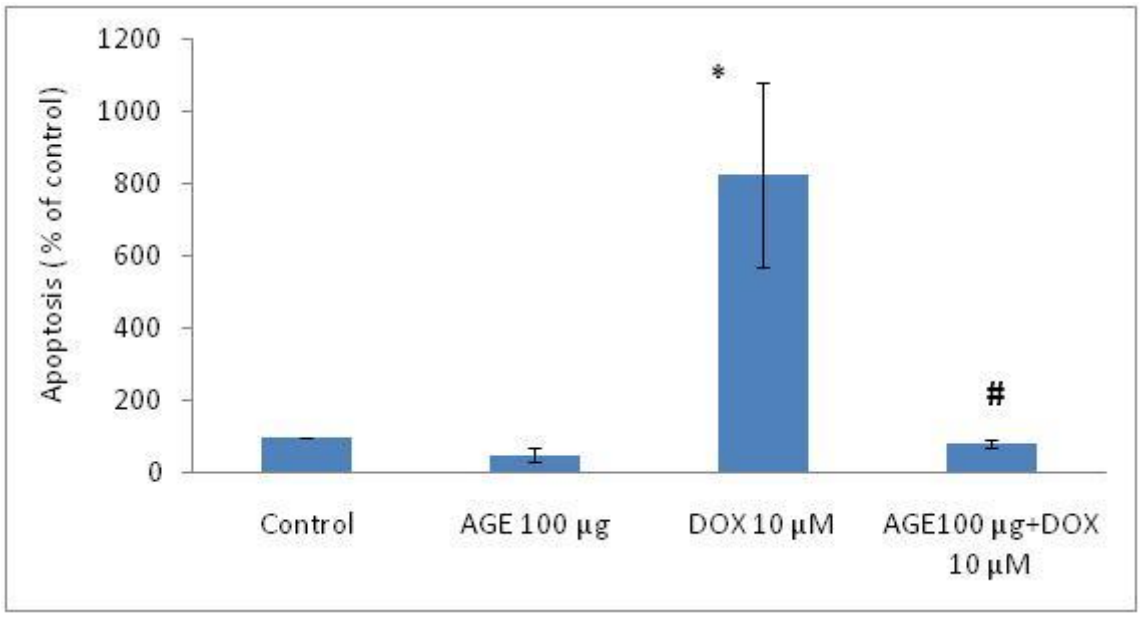


Figure 4.13: The effect of 100µg AGE on the % apoptosis in cardiac myocytes treated with 10µM DOX. After 8 hours' treatment without (control) or with 100 µg AGE, cardiac myocytes were incubated with 10 µM DOX for 24 hours. Mean of apoptotic cell number was plotted by percentage of control. Data are mean ± SEM for a minimum of 3 independent experiments for each condition. *Significantly different from control ($P < 0.05$). # Significantly different from DOX ($P < 0.05$, one way ANOVA with LSD post test).

4.4 Discussion

The current study demonstrated that DOX significantly inhibited cardiac myocyte growth. The concentration of 10 μM DOX was chosen in this study since similar levels are seen transiently in plasma following pharmaceutical use in patients.

The effect of AGE on cardiac myocyte growth was analysed in the present study. Aged garlic extract caused inhibition of the growth of rat cardiac myocytes. So far, there are no studies reporting the effect of AGE on rat cardiac myocytes. Meanwhile, the effect of AGE on other cells has been described. The inhibition of various malignant cell proliferations by AGE has been reported in several studies (Ban *et al.*, 2007; Chu *et al.*, 2007; Herman-antosiewicz *et al.*, 2007; Howard *et al.*, 2007; Omar and Al-Wabel, 2009; Viry *et al.*, 2011). Howard *et al.* (2007) reported that S-allylmercaptocysteine (SAMC) at 300 mg/kg/day is able to inhibit the growth of androgen-independent prostate tumours *in vivo.* Furthermore, SAMC significantly lessened distant metastasis to multiple distant organ sites in a dose-dependent manner. Similarly, Chu *et al.* (2007) investigated the effect of S-allylcysteine (SAC) on CWR22R, a human androgen-independent (AI) prostate cancer xenograft in nude mice. Treatment with SAC caused inhibition of the growth of CWR22R, with no detectable toxic effect on nude mice. A concurrent reduction in serum prostate specific antigen (PSA) level and proliferation rate of xenografts was observed with SAC-induced growth reduction. The observed antiproliferative effect of AGE appeared to be associated with a G2-M cell cycle arrest (Viry *et al.*, 2011).

The treatment of smooth muscle cells with 0.4, 2, 10, and 50 mg of AGE for 24 hours revealed a dose-dependent inhibition of proliferation ($p<0.01$). However, cells were morphologically normal in the presence of AGE even after 48 hours (Efendy *et al.*, 1997).

The present study demonstrated that AGE inhibited DOX- induced oxidative stress which was appearant as increased 8-isoprostane in rat cardiac myocytes culture media. The increase in 8-isoprostane production caused by DOX was greatly reduced in AGE+DOX-treated cells. Oxidative stress is one of the major factors involved in the pathogenesis of DOX-induced cardiotoxicity (Oliveira *et al.*, 2004; Oliveira *et al.*, 2006; Barry *et al.*, 2007; Ozdogan *et al.*, 2011).

Several reports found that AGE has a significant antioxidant activity (Drobiova *et al.*, 2009; Arguello-Garcia *et al.*, 2010; Luo *et al.*, 2010; Heidarian *et al.*, 2011; Nencini *et al.*, 2011; Ponnusamy and Pari, 2011; Ray *et al.*, 2011; Rojas *et al.*, 2011). Rojas *et al.* (2011) found that a dose of 125 mg/kg i.p. of SAC produced a decrease in superoxide radical production and blocked (100% of protection) of lipid peroxidation in mice. Dillon *et al.* (2002) studied the effects of dietary supplementation with AGE on the plasma and urine concentrations of the 8-iso-prostaglandin $F_{2\alpha}$ in smoking and non-smoking subjects. Dietary supplementation with AGE for 14 day significantly lowered plasma and urine concentrations of 8-iso-prostaglandin $F_{2\alpha}$ by 29% and 37% in non-smokers and by 35% and 48% in smokers.

This study has found that Caspase-3 activity was significantly increased in DOX-treated cardiac myocytes. The Pre-treatment of cells with AGE significantly reduced DOX-induced caspase-3 activation. The activation of caspase-3 is a key mechanism in apoptosis. Caspase-3 activity first becomes detectable early in apoptosis, continues to increase as cells undergo apoptosis, and rapidly diminishes in late stages of apoptosis. The induction of caspase-3 activity is an early marker of cells undergoing apoptosis. The activation of Caspase-3 in DOX-induced cardiotoxicity has been documented in previous studies (Gilleron *et al.*, 2009; Kassab, 2009).

Pointon *et al.* (2010) reported that Caspase-3 was increased with acute DOX and up to 50--fold over control by 30 minutes post-dose in the heart of mice treated with a single dose of DOX at 15 mg/kg. Another study by Frias *et al.* (2010) demonstrated that the treatment of neonatal rat ventricular cardiac myocytes with DOX at 0.5 μM significantly induced caspase-3 activation in cells approximately 100 fold above control. Jackson *et al.* (2002) reported that AGE inhibited caspase-3 in neuronal cells in a dose-dependent manner.

In this study, a significant increase in active p53 was demonstrated following the treatment of cardiac myocytes with DOX. Meanwhile, the pre-incubation of cells with AGE produced a noticeable reduction in active p53.

It has been demonstrated that the exposure of cardiac myocyte to 1 μM of DOX for 24 hours resulted in a significant increase in phospho-p53 followed by cleaved Caspase-3 expression and apoptotic cell death (Ueno *et al.*, 2006). Sardão *et al.* (2009) found that the treatment of cardiac myocyte with 1 μM of DOX for 24 hours caused a significant increase in p53 nuclear fluorescence intensity and caspase-3 activity.

The present study demonstrated that the pre-treatment of cardiac myocytes with AGE decreased the percentage of apoptotic cells in DOX-treated cells. Green and Leeuwenburgh (2002) investigated the effects of DOX 10 μM on rat cardiac H9c2 cells. The exposure of H9c2 cells to 10 μM of DOX for 20 hours resulted in a 45% increase in apoptotic cells and a significant increase in active caspase-3.

Damage to DNA, oxidative mitochondrial injury, and nuclear translocation of p53, are all implicated in the cardiotoxic effects of doxorubicin (Chua *et al.*, 2006; L'Ecuyer *et al.*, 2006; lefrak *et al*., 1973; Doroshow, 1980). The transcription factor p53 is one of the proteins involved in the cell responses to DNA damage (Gomez-Lazaro *et al.*, 2004).

In the current study, DOX-induced p53 activation may be caused by extracellular signal-regulated kinases (ERKs). Agents that damage DNA usually activate ERKs (Schweyer *et al.*, 2004, Kim *et al.*, 2005). Since p53-dependent apoptosis is affected by the mitogen- activated protein kinase (MAPK) cascade, the increased activity of p53 and cell death produced by DOX in rat cardiac myocytes may be generated by ERK1/2 (Persons *et al.*, 2000, Pearson *et al.*, 2001, Alkhalaf and Jaffal, 2006, Brown and Benchimol, 2006). Liu *et al.*, 2008 demonstrated that there is increased activation and nuclear translocation of ERK1/2 and p53 associated with cytotoxicity of DOX in neonatal rat cultured cardiac myocytes. Furthermore, they showed that caspase-3 is activated in DOX-treated cardiac myocytes

The antiapoptotic effect of AGE in this study could be due to an inhibitory effect of on the phosphorylation of ERK 1/2 molecules. It has been shown that SAC suppress MAPK/ERK signaling pathway in human oral squamous cancer (Tang *et al.*, 2009). Another study by (Kim *et al.*, 2006) reported a neuroprotective effect of SAC through suppression of ERK signaling pathway. Peng *et al.*, 2002 showed that AGE and SAC protected rat pheochromocytoma (PC12) cell line from amyloid-beta peptide-induced apoptosis through reduced caspase-3 activation, DNA fragmentation, poly (ADP-ribose) polymerase (PARP) cleavage.

In conclusion, the results of the current study demonstrate that AGE protects rat cardiac myocytes against DOX-induced apoptosis in a dose-dependent manner using PI/DAPI staining, active p53, and caspase-3 activity assays. Accordingly, it is stated that AGE may be effective in reducing DOX-induced cardiotoxicity.

Chapter 5 The effect of doxorubicin on oxidative stress and antioxidant gene expression in rat cardiac myocytes in the presence and absence of aged garlic extract.

5.1 Introduction

It has been established that DNA damage is a consequence of the effective anti-cancer drug DOX. The drug is predominantly powerful when used against those tumours which are rapidly proliferative (Lee and Byfield, 1976, Kanter and Schwartz, 1979, Singal *et al.*, 1987). In contrast, cardiac myocytes are only slightly replicative cells known to be unaffected by such antimitotic mechanisms. In this regard, free radical generation adds to the cardiotoxic impacts induced by DOX (Olson and Mushlin, 1990, Fisher, 1994). Notably, DOX is known to generate free radicals directly. Moreover, through redox cycling, DOX is a strong chemical catalyst for the production of oxygen radicals (Doroshow, 1983, Powis, 1989, Olson and Mushlin, 1990). Furthermore, decreases in the quantity of endogenous antioxidants have been demonstrated following DOX treatment (Singal *et al.*, 1997).

The oxidative damage induced by DOX is complicated affecting lysosomes, microfibrils mitochondria and sarcoplasmic reticulum (Ogura *et al*., 1991, Myers *et al*., 1977, Mimnaugh *et al*., 1985, Singal *et al*., 1987). Eventually, these intracellular modifications result in increased apoptosis in cardiac myocytes.

The conversion of arachidonic acid to prostaglandin H2 (PGH2), the common intermediate in prostaglandin, prostacyclin and thromboxane synthesis, is catalyzed by the cyclooxygenases (Coxs). Variety of ligands, including tumor promoters, growth factors, cytokines, endotoxins and mitogens stimulate the transcription of prostaglandin-

endoperoxide synthase 2 ,Cox-2, (Ptgs2) gene which is an immediate-early gene (Herschman, 2004). Control of Cox gene expression differs in different cell types and even between the same cell types in different species. There is variation in the signal transduction pathways for Cox-2 induction depending on the stimulus and cell type (Kang *et al*., 2007). Cardiac myocytes are protected from oxidant injury by Cox-2-dependent prostaglandins (Adderley and Fitzgerald, 1999).

Hydrogen peroxide and organic hydroperoxides are scavenged by the primary antioxidant enzymes glutathione peroxidases (Gpxs). Glutathione peroxidases guard biomembranes and cellular components against oxidative stress (Brigelius-Flohé, 1999). Glutathione peroxidase acts as a peroxynitrite reductase. The oxidation and nitration reactions caused by peroxynitrite are prevented by Gpx (Sies *et al*., 1997).

There is variation in the primary structure and localization of Gpx isoforms. The first of the Gpx family to be discovered was the cytosolic-mitochondrial Gpx1 (cGpx), a selenium-dependent enzyme. The gastrointestinal Gpx2 is a cytosolic enzyme present mainly in the epithelium of the gastrointestinal tract (Chu *et al*., 2004). Extracellular plasma Gpx (pGpx, or Gpx3) is located mostly in the kidney. Phospholipid hydroperoxide glutathione peroxidase (PhGPx or Gpx4) is found in most tissues. Epididymis-specific secretory Gpx is Gpx5 or eGPx (Brigelius-Flohé, 1999, Brigelius-Flohe and Flohe, 2003). Less is known about Gpx6 and Gpx7.

A new family of antioxidant enzyme is the Peroxiredoxins (PRDXs) which are expressed in all biological kingdoms (Butterfield *et al*., 1999). There are six PRDX isoforms identified and characterized in mammals (Chae *et al*., 1999, Wood *et al*., 2003). They have important role in eliminating H_2O_2 and neutralizing other oxidizing molecules (Schroder and Ponting,

1998). Peroxiredoxin 5 (Prdx5) is the last member to be recognized amidst the six mammalian peroxiredoxins. In mammals, Prdx5 is a unique atypical 2-Cys peroxiredoxin. It is commonly found in tissues and has large subcellular distribution. Peroxiredoxin 5 is mainly a cytoprotective antioxidant enzyme which acts against exogenous or endogenous peroxide attacks (Kropotov *et al*., 2006). It has been reported that Prdxs suppress apoptosis mediated by hydrogen peroxide (Kang *et al*., 2005).

In mitochondria, the process of nutritional substrates oxidation is combined with ATP synthesis. Electrons move through the respiratory chain during substrate oxidation, which, at the same time, expels protons from the mitochondrial matrix, transporting them into the intermembrane space (Mitchell, 1961). Uncoupling proteins (UCPs), present in the mitochondrial inner membrane intercede uncoupling or proton leak. The uncoupling proteins established so far comprise UCP1, UCP2, UCP3 and UCP4, as well as brain mitochondrial carrier protein (BMCP1, also known as UCP5). In this regard, a number of researches state that UCP2 and UCP3 are both linked with slight uncoupling and decreased mitochondrial superoxide production, thereby protecting against oxidative damage (Echtay, 2007, McLeod *et al*., 2005, Nadtochiy *et al*., 2006, Cannon *et al*., 2006)

Cytoglobin (Cygb) is recognised as a member of the vertebrate globin family, which is produced amongst different tissues at various levels (Fordel *et al*., 2004, Shigematsu *et al*., 2008, Pesce *et al*., 2002). Researcher have suggested that cytoglobin up-regulated by hydrogen peroxide plays a protective role in oxidative stress (Li *et al*., 2007).

The mechanism of action of DOX-induced oxidative stress is still unclear. In this study the exposure of cardiac myocytes to DOX was examined to assess oxidative stress and

antioxidant defence. Genes that are known to be upregulated by oxidative stress could be expected to be upregulated when cells are exposed to DOX.

The current study aimed to investigate the effect of doxorubicin on oxidative stress and antioxidant gene expression in rat cardiac myocytes in the presence and absence of AGE. The objectives were:

To investigate the expression of 84 genes relating to oxidative stress using the rat oxidative stress and antioxidant defence RT² Profiler™ PCR Array.

To investigate the expression of some chosen genes form the RT² Profiler™ PCR array using real-time reverse transcription PCR (RT^2 qPCR) and semi-quantitative RT- PCR.

5.2 Materials and methods

Effect of doxorubicin, aged garlic extract or both on gene expression in cultured rat cardiac myocytes

Rat cardiac myocytes were cultured as described in section 2.13. Cells were incubated with 100 µg AGE for 8- hours followed by incubation with 10 µM DOX for 4 hours. Treatment of cells with DOX for 4 hours were chosen after pilot experiments at 1, 4 and 24 hours. The best time for detection of gene expression was after 4 hours exposure to DOX. After this incubation, cells were collected by centrifugation (1000 g for 5 minutes) and parameters were measured as described in Chapter 2. RNA extraction for polymerase chain reaction (PCR) array and real- time PCR was conducted by the method described in section 2.17. cDNA synthesis was conducted by the method described in section 2.18. Rat oxidative stress and antioxidant defence PCR array were measured by the method described in section 2.19. Real-time reverse transcription PCR (RT^2 qPCR) were measured by the methods

described in section 2.20. Reverse transcription-PCR (RT-PCR) were measured by the methods described in section 2.21. Agarose gel electrophoresis of DNA was measured by the methods described in section 2.22.

5.3 Results

Gene array results

In order to minimize false positive results and generate a reliable shortlist, only those genes upregulated or downregulated >2-fold were selected. Of 84 genes analyzed only 6 genes were observed to be changed when DOX-treated cells were pre-incubated with AGE on the gene array (Table 5.1).

Table 5.1. illustrates the number of genes that were expressed in cardiac myocytes., such as Prostaglandin-endoperoxide synthase 2 or Cox-2 (Ptgs2), Glutathione peroxidase 7(Gpx7), Peroxiredoxin 5 (Prdx5), Uncoupling protein 3 (mitochondrial, proton carrier) (Ucp3), Cytoglobin (Cygp), and Glutathione peroxidise 2 (Gpx2) genes.

Validation of microarray results

In order to validate the results of array analysis the technique of real-time reverse transcription qPCR (RT^2 qPCR) or semi-quantitative PCR were used. Products of PCR amplification of cDNA prepared from RNA extracted from cardiac myocytes was compared on an agarose gel after a fixed number of PCR cycles.

Standard curve

For each primer pair, standard curves were constructed to calculate the PCR efficiency. To do this, cDNA from cardiac myocytes expressing the target gene (Table 5.1) was serial

diluted and amplified, to produce standard curves expressing a linear relationship between template quantity and target gene expression (Figure 5.1).

Dissociation curves

In order to ensure that any increase in fluorescence was due to cDNA amplification for the gene of interest and not caused by non-specific products or primer dimer artefacts, dissociation curves were plotted for all PCR products. Dissociation curves for all genes analysed were shown to be specific with only a single peak at the melting temperature of the PCR product. Figure 5.2 shows an example of the dissociation curve.

Housekeeping gene selection

Real-time qPCR is a sensitive and accurate technique for measuring target mRNA expression. Normalisation of the results to compensate for differences in the purity and concentration of the samples is a key step of this technique. Endogenous reference genes or housekeeping genes are the most commonly used normalisers in real-time PCR.

Preferably, housekeeping genes should be ubiquitously expressed at similar levels in all samples and experimental conditions. Two housekeeping genes were selected for validation hypoxanthine phosphoribosyltransferase 1 (Hprt1), and lactate dehydrogenase A (Ldh1). Since Hprt1 and Ldh1 displayed similar levels of expression, all subsequent target gene expression analyses were normalised using the average of the expression of Hprt1, and Ldh1.

Figure 5.3 demonstrate approximately equal expression of the housekeeping gene Hprt1 in all samples. Hence it was chosen for normalization of other genes in semi-quantitative reverse transcription PCR.

Table 5.1:The effect of 100 µg AGE pre-incubation on the oxidative stress mediated gene expression in rat cardiac myocytes after 4 hours incubation with DOX 10 µM.

Gene description	symbol	Fold change		
		AGE/CT	DOX/CT	AGE+DOX/CT
Prostaglandin-endoperoxide synthase 2	Ptgs2	1	-5	-5
Glutathione peroxidase 7	Gpx7	1	-5	1
Peroxiredoxin 5	Prdx5	1.25	-5	1.29
Uncoupling protein 3 (mitochondrial, proton carrier)	Ucp3	2.74	4.35	1.25
Cytoglobin	Cygb	1.69	6.97	6.6
Glutathione peroxidise 2	Gpx2	1.31	2.95	4.22

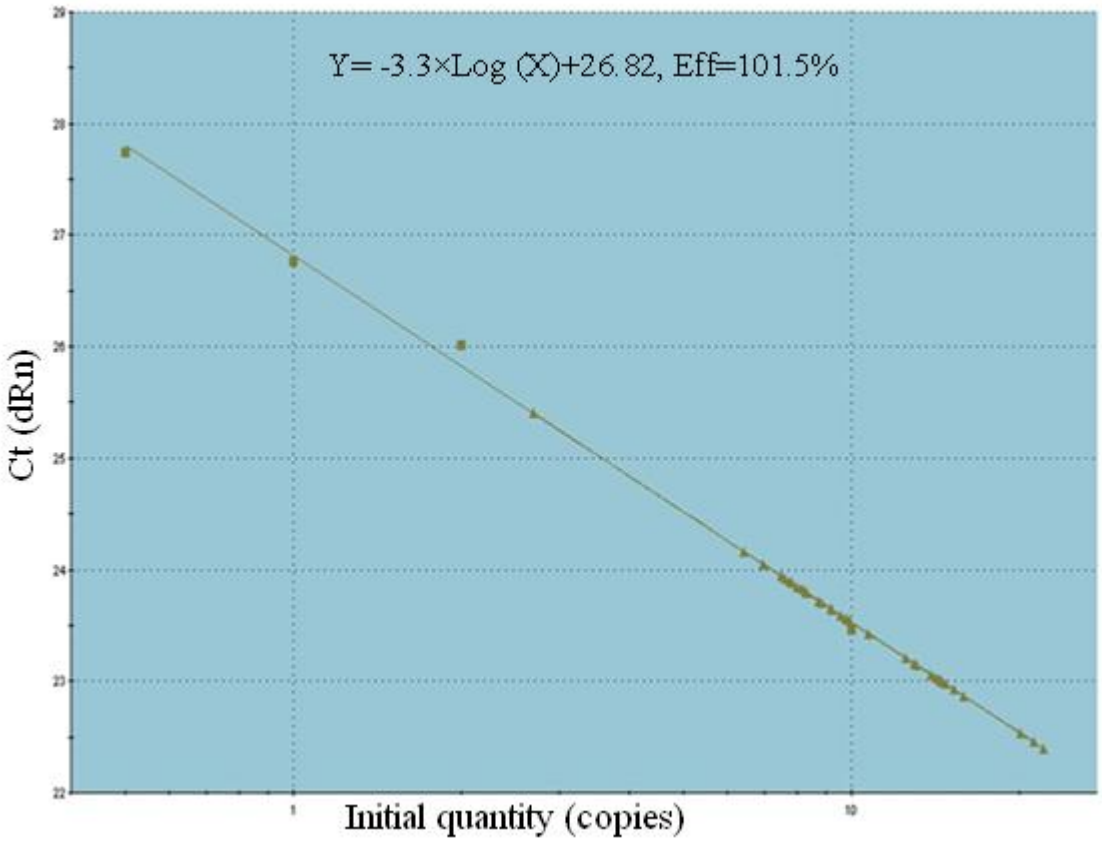


Figure 5.1: A representative diagram of standard curve for primer pairs for real-time qPCR. Each reaction was carried out in triplicate.

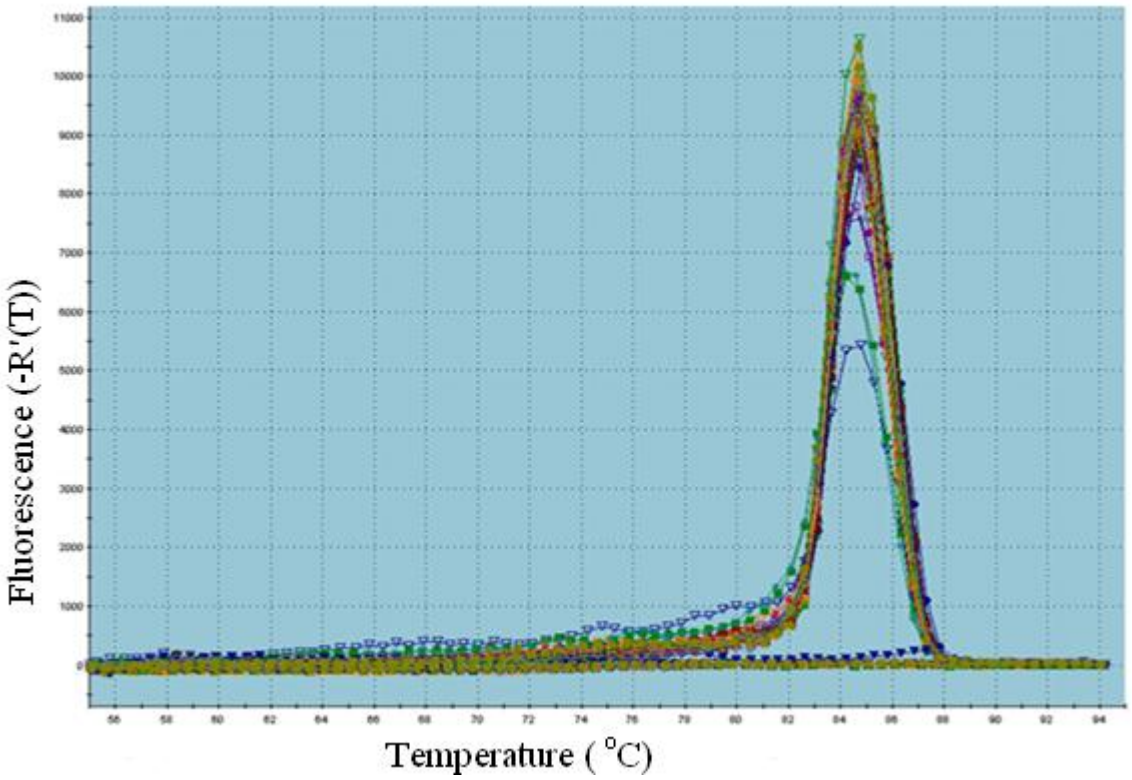
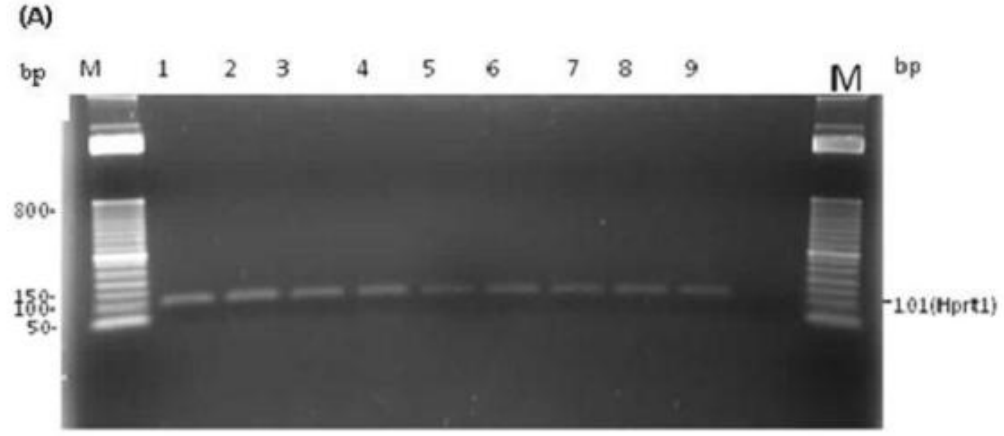
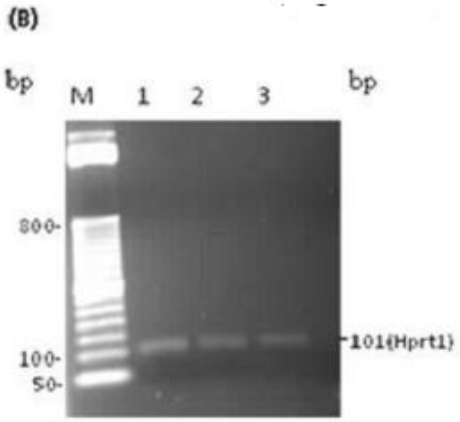


Figure 5.2: A representative diagram of dissociation curve in real-time qPCR



M: 50 bpDNA ladder
Lanes: 1-3 control
Lanes: 4-6 DOX
Lanes: 7-9 AGE
Lane: 10 blank (negtative control)



M: 50 bpDNA ladder
Lane: 1 control
Lanes: 2/3 AGE+DOX

Figure 5.3: Gel showing semi-quantitative RT-PCR results of house keeping Hprt1 gene expression in rat cardiac myocytes. PCR products were loaded on 2% agarose gel and stained with ethidium bromide. 50bp DNA ladder was used as marker.

Prostaglandin-endoperoxide synthase 2 or Cox-2 (Ptgs2)

The changes in the mRNA levels of Cox-2 (Ptgs2) were investigated by semi-quantitative RT-PCR and real time-qPCR. Semi-quantitative RT-PCR showed a single product of the expected size (120 base pairs) in all samples (Figure 5.4). The intensity was less in DOX and AGE+DOX-treated cells compared to the AGE-treated cells (Figure 5.5). Analysis of Ptgs2 expression using real-time qPCR (Figure 5.6) supported the pattern in expression observed by semi-quantitative reverse transcription PCR. Doxorubicin and AGE+DOX-treated cells each had relative expression of 0.101 and 0.094 respectively, with that of AGE-treated cells reaching 1.86.

Glutathione peroxidase 7

Semi-quantitative RT- PCR and real-time qPCR both showed Gpx7 expression was higher in DOX-treated cells compared to AGE+DOX treated cells (Figures 5.7-5.9). Analysis of Gpx7 expression using real-time qPCR demonstrated that DOX and AGE+DOX-treated cells had relative expression of 1.35 and 1.29 respectively, with that of AGE-treated cells showing 0.93 (Figure 5.9).

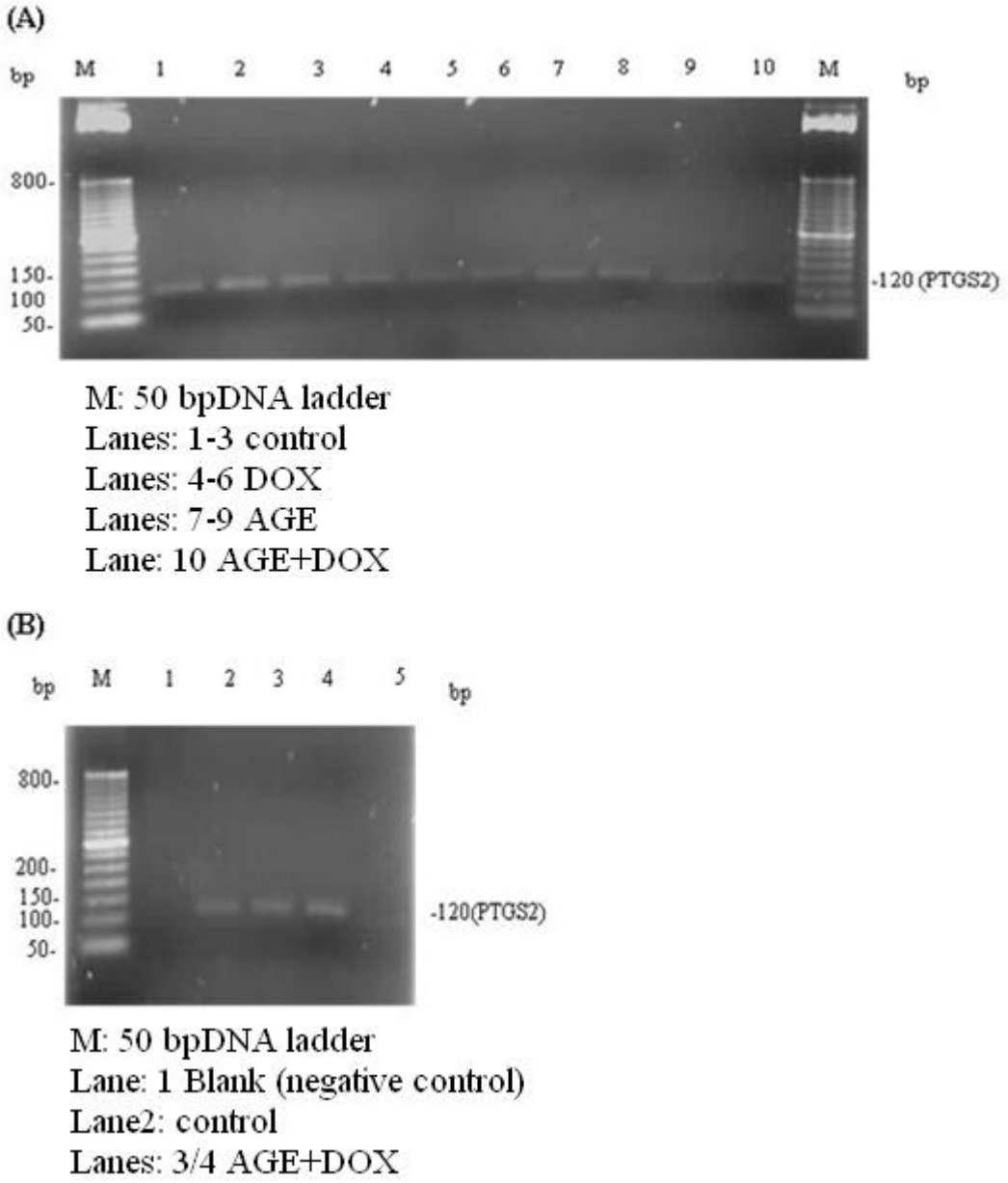


Figure 5.4: Gel showing semi-quantitative RT-PCR results of Ptgs2 (Cox-2) gene expression in rat cardiac myocytes. PCR products were loaded on 2% agarose gel and stained with ethidium bromide. 50bp DNA ladder was used as marker.

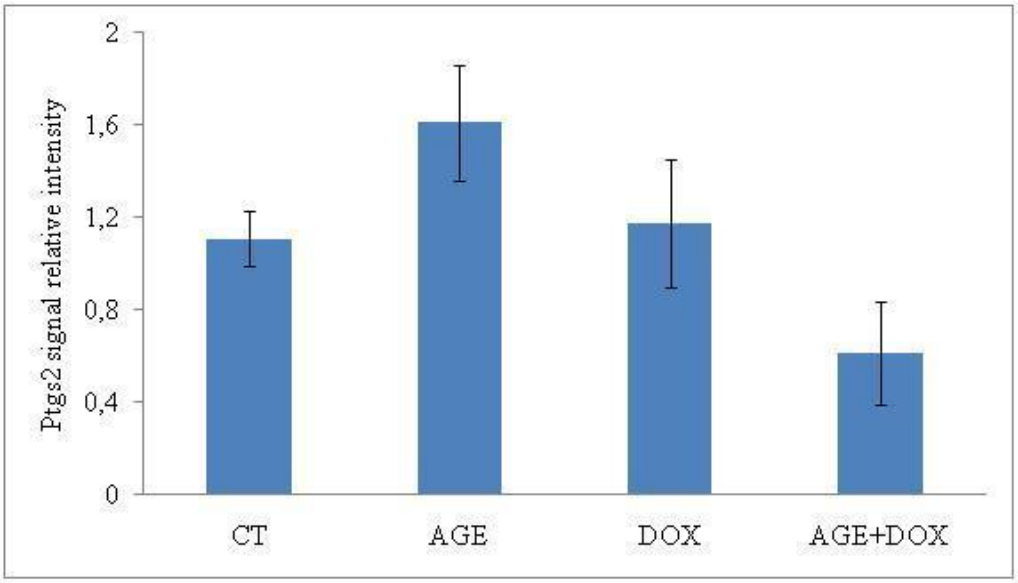


Figure 5.5:Semi-quantitative RT- PCR analysis of Ptgs2 expression in cardiac myocytes. Results represent mean ± SEM (n=3). No significant differences between groups ($p>0.05$, one way ANOVA).

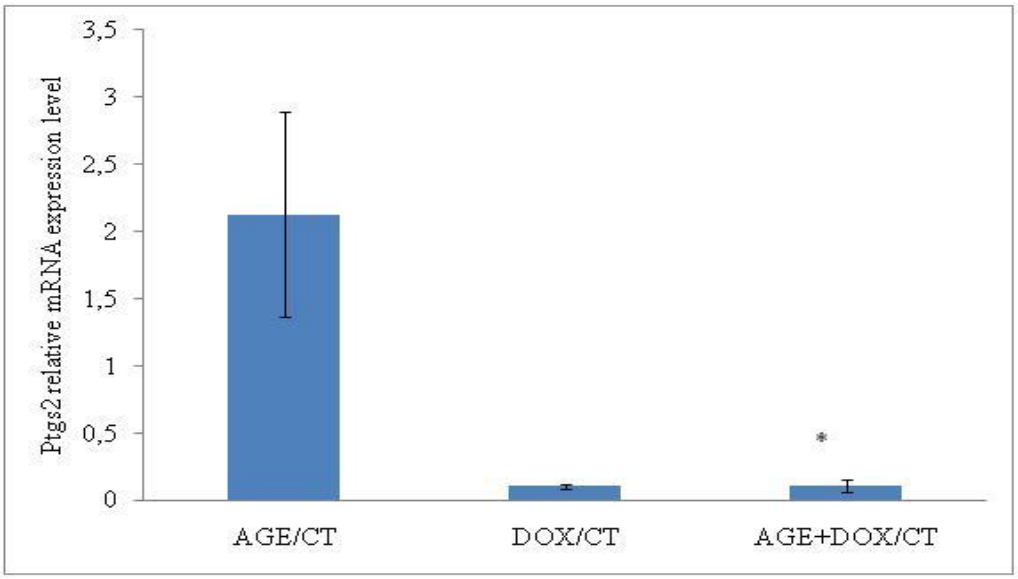


Figure 5.6: The mRNA expression levels of Ptgs2 gene quantified by real-time qPCR. Each column represents the mean ± SEM (N=3). * Significantly different from AGE -treated cells ($p< 0.05$, one way ANOVA with LSD post test).

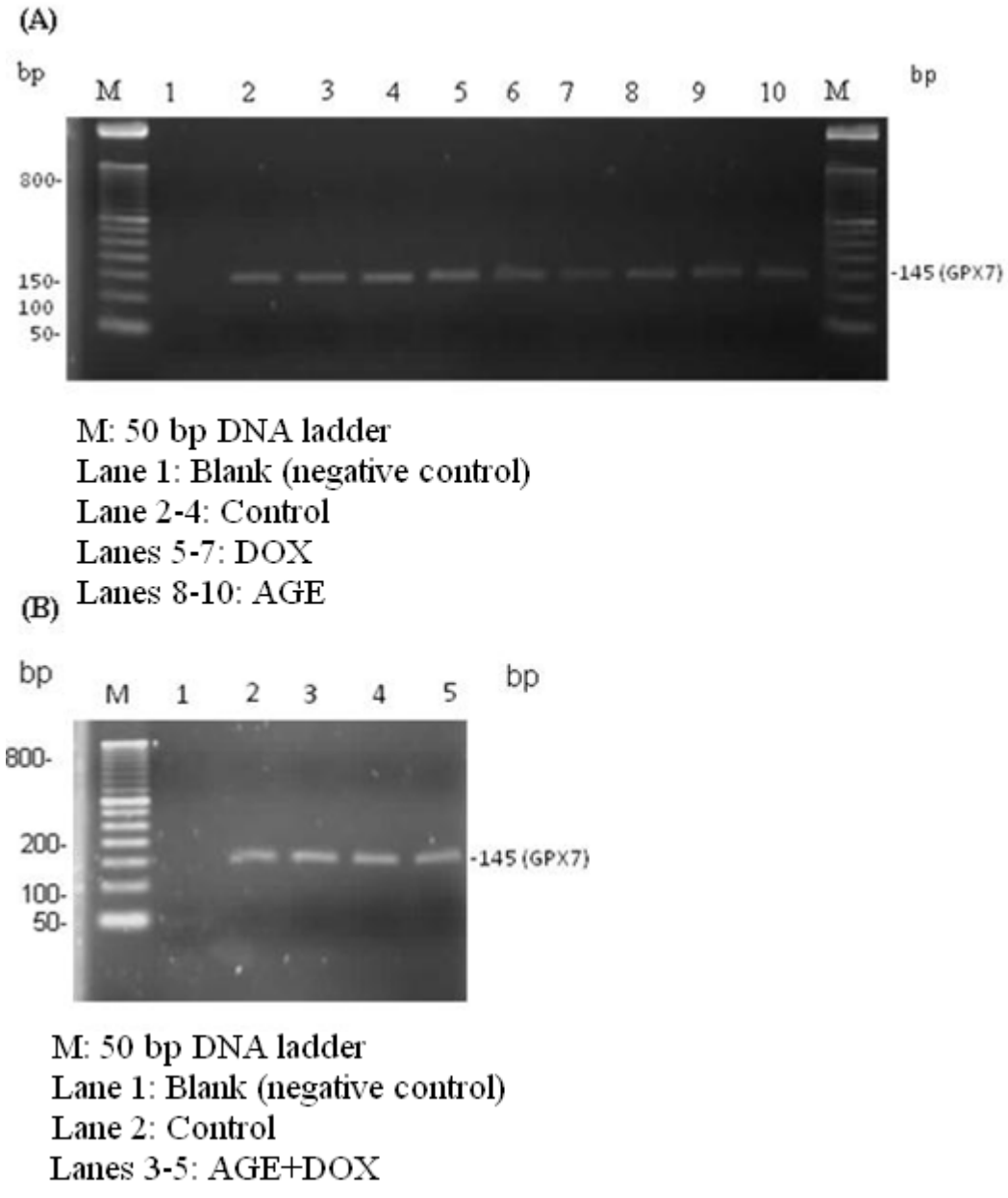


Figure 5.7: Gel showing semi-quantitative RT- PCR results of Gpx7 gene expression in rat cardiac myocytes. PCR products were loaded on 2% agarose gel and stained with ethidium bromide. 50bp DNA ladder was used as marker.

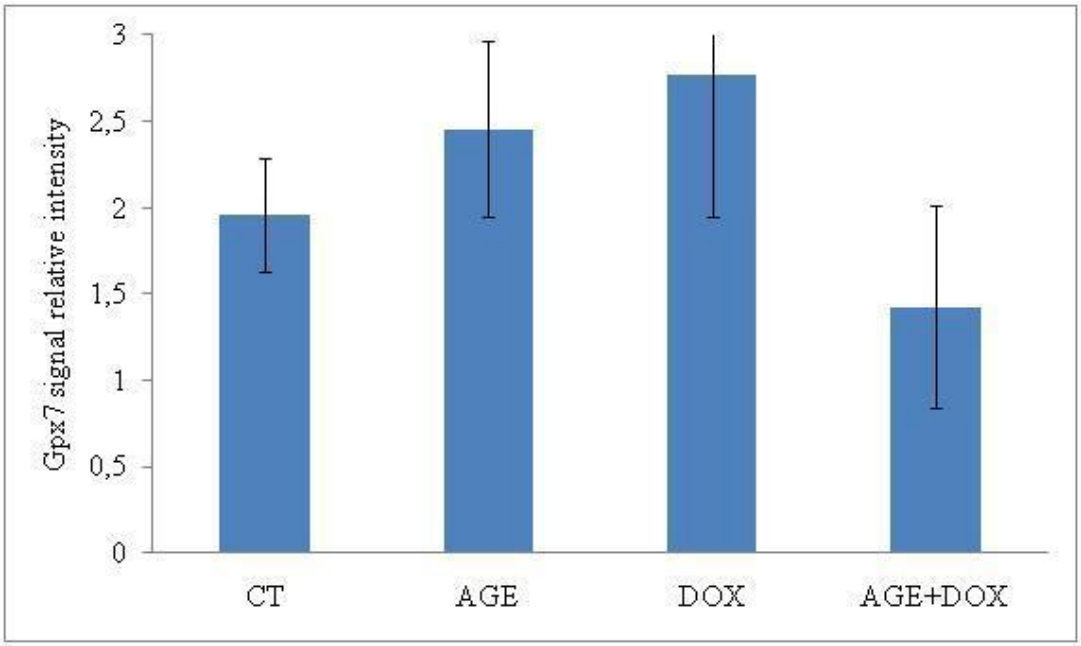


Figure 5.8:Semi-quantitative RT- PCR analysis of Gpx7 expression in rat cardiac myocytes. Results represent mean ± SEM (n=3). No significant differences between groups ($p>0.05$, one way ANOVA).

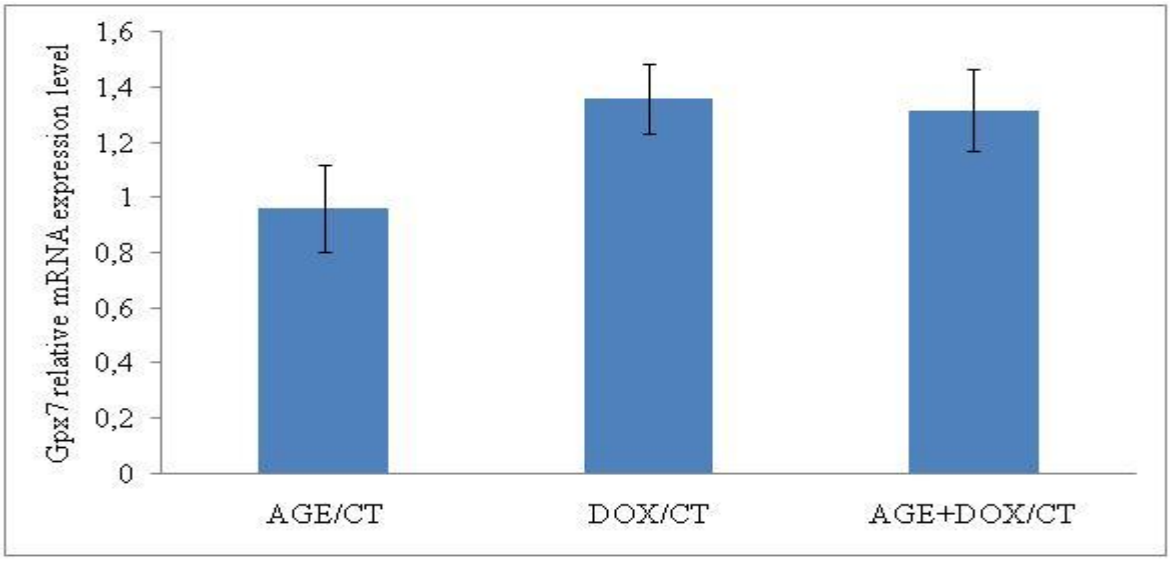


Figure 5.9: The mRNA expression levels of Gpx7 quantified by real-time qPCR. Each column represents the mean mean ± SEM (N=3). No significant differences between groups ($p>0.05$, one way ANOVA).

Peroxiredoxin 5

Semi-quantitative reverse transcription PCR demonstrated that Prdx5 expression was higher in DOX-treated cells compared to AGE+DOX-treated cells, while in real-time qPCR, DOX-treated cells showed a lower expression level than AGE+DOX-treated cells (Figures 5.10, 5.11). Analysis of Prdx5 expression using real-time qPCR showed that DOX and AGE+DOX-treated cells had relative expression of 1 and 1.27 respectively, with that of AGE-treated cells showing 1.49 (Figure 5.12). Semi-quantitative RT- PCR is in agreement with microarray.

Uncoupling protein 3 (mitochondrial, proton carrier) (Ucp3)

Semi-quantitative RT-PCR did not reveal Ucp3 product in all samples (Figure 5.13). Similarly, real-time qPCR demonstrated absent gene expression in contol cells. In microarray, DOX-treated cells showed higher Ucp3 expression than AGE and AGE+DOX-treated cells reaching 4, 35 and that of AGE and AGE+DOX-treated cells were 2, 74 and 1,25 respectively (Table 5.1).

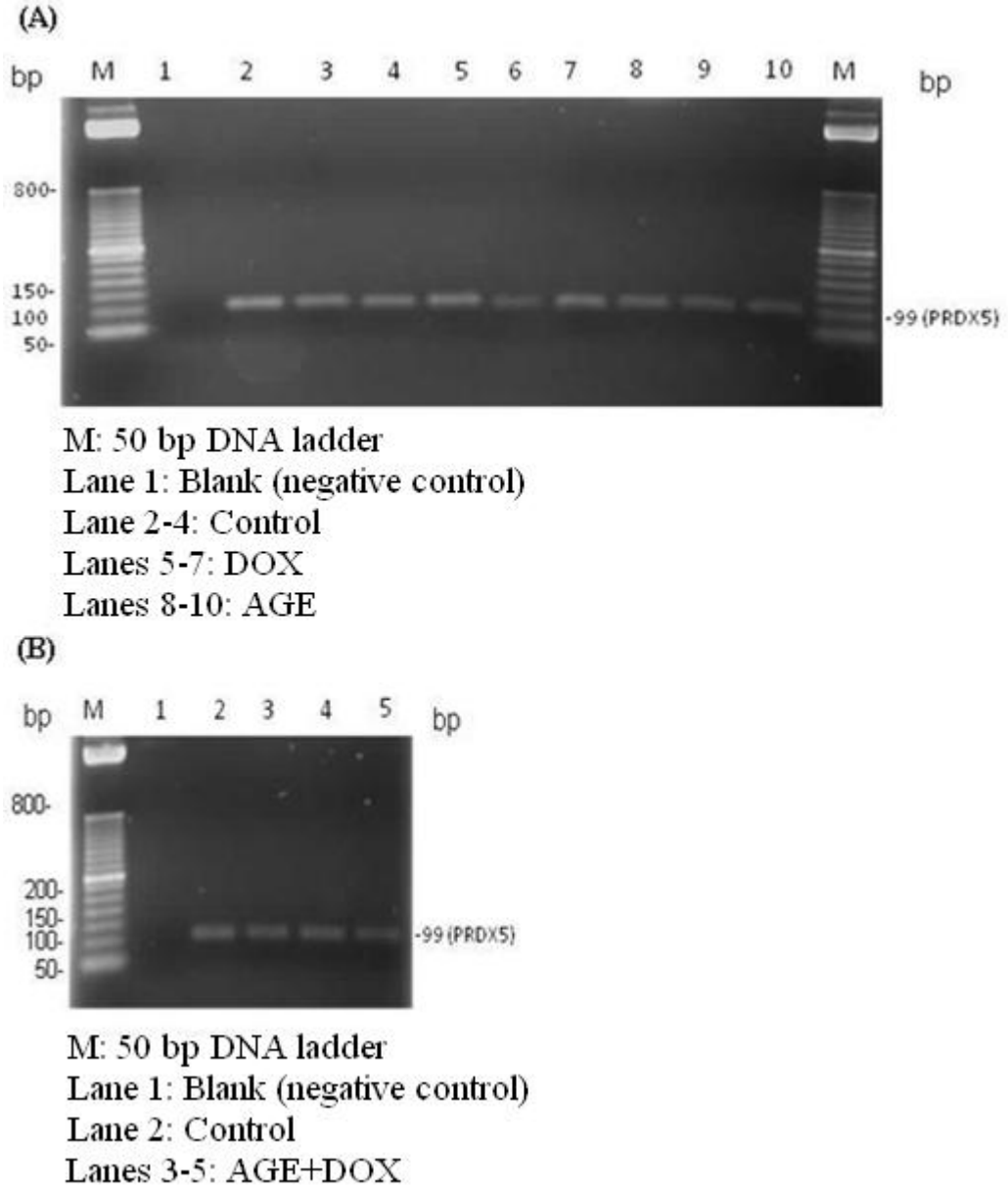


Figure 5.10: Gel showing semi-quantitative RT- PCR results of Prdx5 gene expression in rat cardiac myocytes. PCR products were loaded on 2% agarose gel and stained with ethidium bromide. 50bp DNA ladder was used as marker.

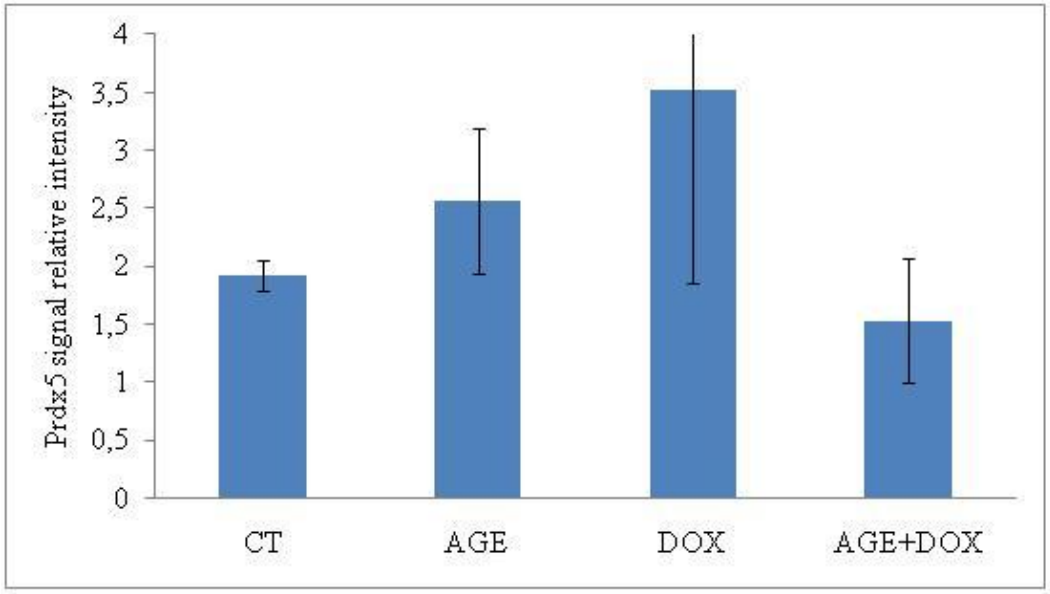


Figure 5.11: Semi-quantitative RT- PCR analysis of Prdx5 expression in cardiac rat myocytes. Results represent mean ± SEM (n=3). No significant differences between groups ($p>0.05$, one way ANOVA).

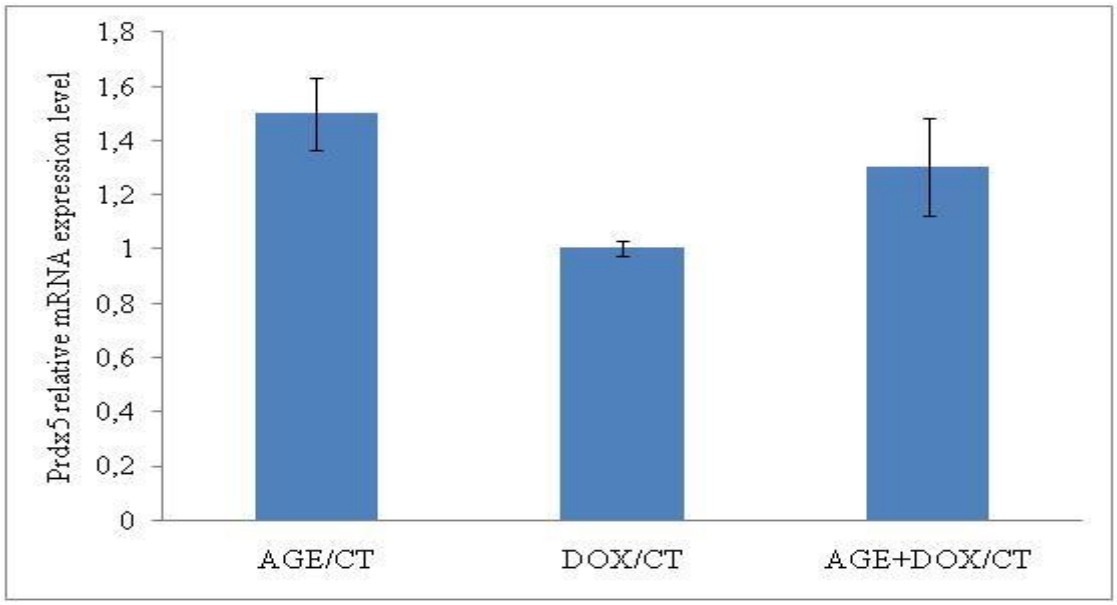


Figure 5.12: The mRNA expression levels of Prdx5 gene quantified by real-time qPCR. Each column represents the mean ± SEM (N=3). No significant differences between groups ($p>0.05$, one way ANOVA).

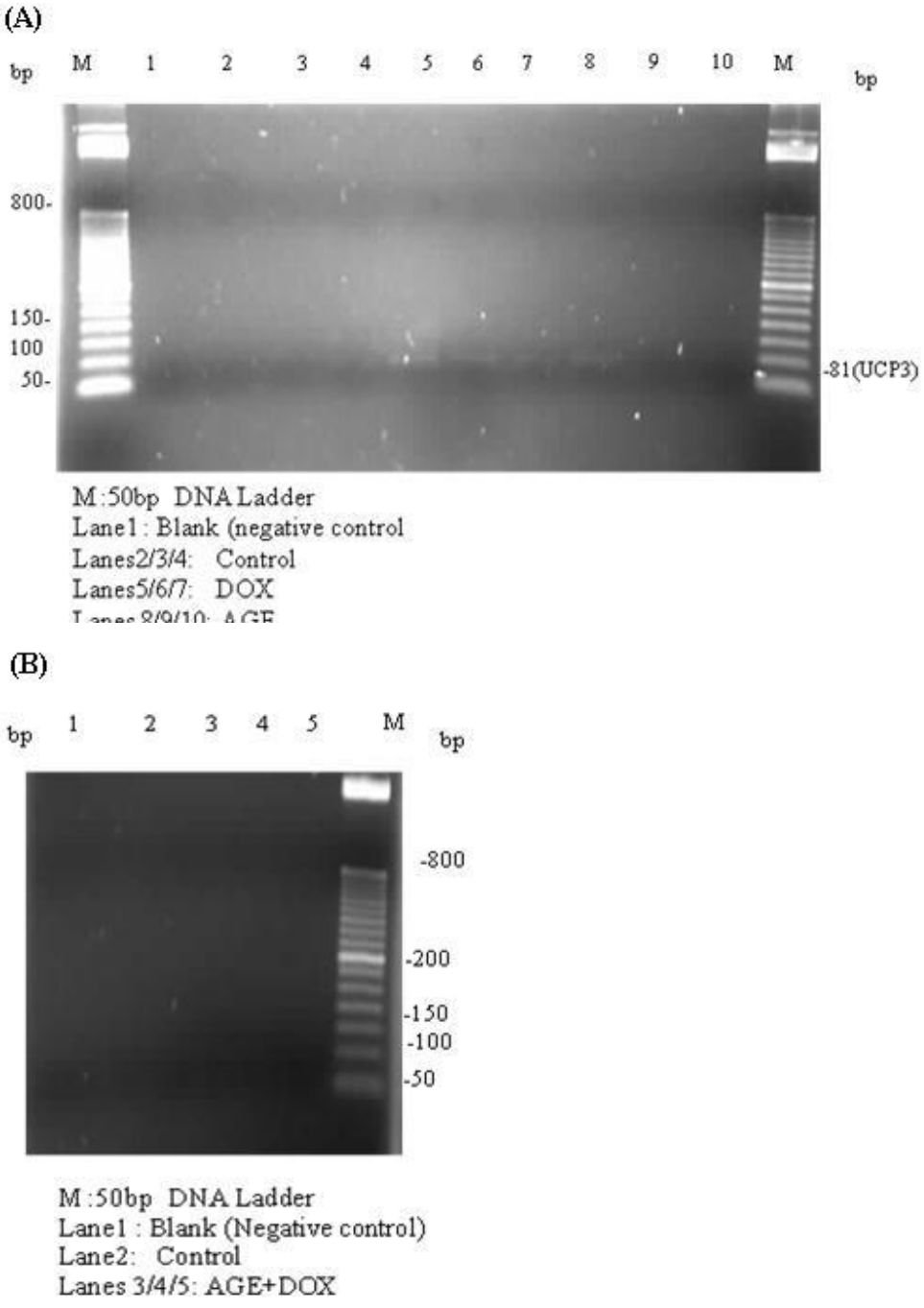


Figure 5.13: Gel showing semi-quantitative RT-PCR result of Ucp3 gene expression in rat cardiac myocytes. PCR products were loaded on 2% agarose gel and stained with ethidium bromide. 50bp DNA ladder was used as marker.

Cytoglobin

Semi-quantitative RT- PCR showed a single product of the expected size, 130 base pairs, in all samples (Figure 5.14). Doxorubicin-treated cells showed higher Cybg expression than AGE and AGE+DOX-treated cells (Figure 5.15) whilst real-time qPCR displayed higher Cygb expression in AGE+DOX- treated cells reaching 3,32 and that of DOX and AGE-treated cells were 1,45 and 0,97 respectively (Figure 5.16).

Glutathione peroxidase 2

Both the semi-quantitative RT- PCR and real-time qPCR demonstrated expression of Gpx2 to be higher in DOX-treated cells compared to AGE and AG+DOX-treated cells (Figures 5.17-5.19). Cells treated with DOX showed relative expression of Gpx2 reaching 42, 22 whilst that of AGE and AGE+DOX-treated cells were 0,781 and 5, 60 respectively Figure (5.19)

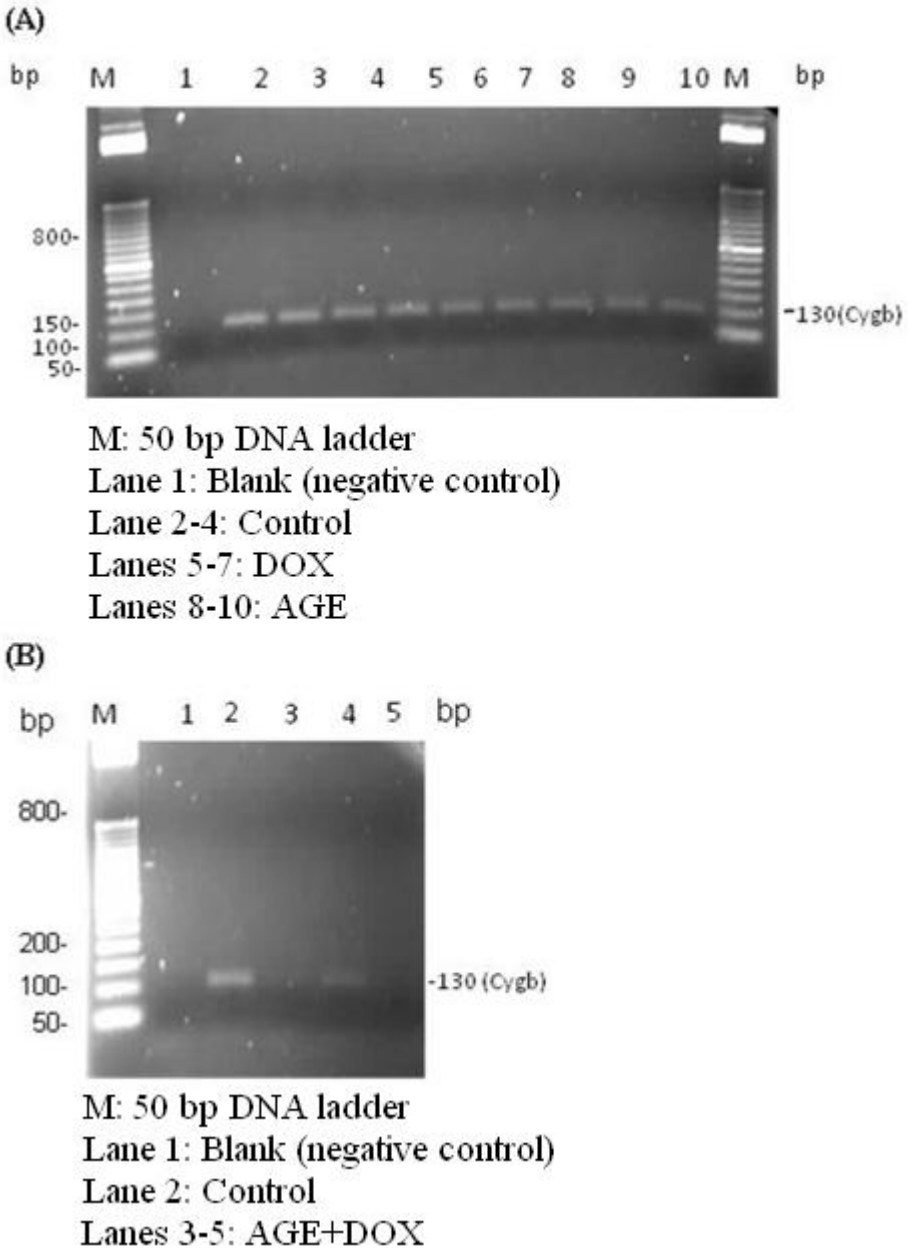


Figure 5.14: Gel showing semi-quantitative RT- PCR results of Cygb gene expression in rat cardiac myocytes. PCR products were loaded on 2% agarose gel and stained with ethidium bromide. 50bp DNA ladder was used as marker.

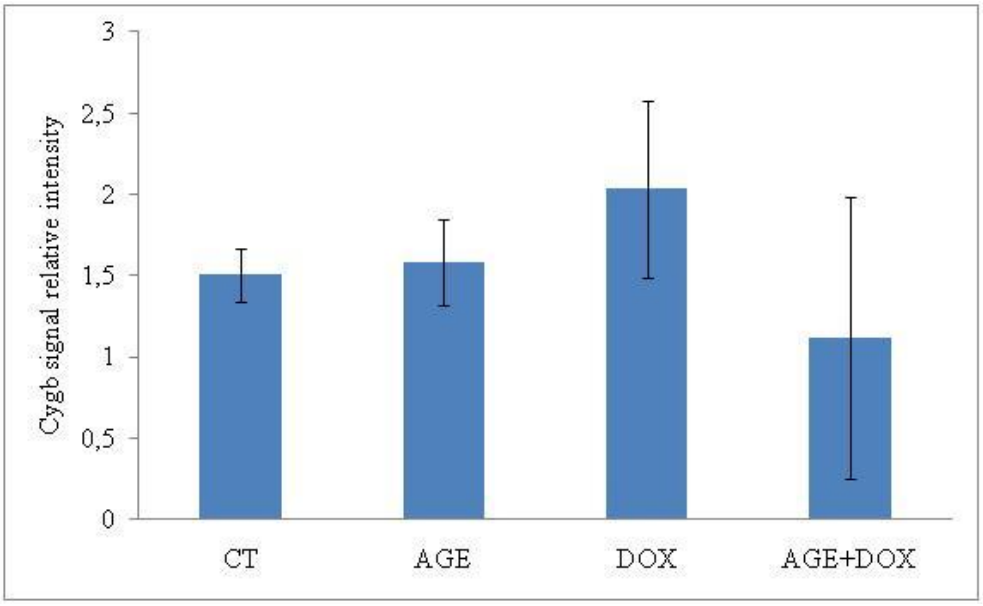


Figure 5.15:Semi-quantitative RT- PCR analysis of Cygb expression in rat cardiac myocytes. Results represent mean ± SEM (n=3). No significant differences between groups (p>0.05, one way ANOVA).

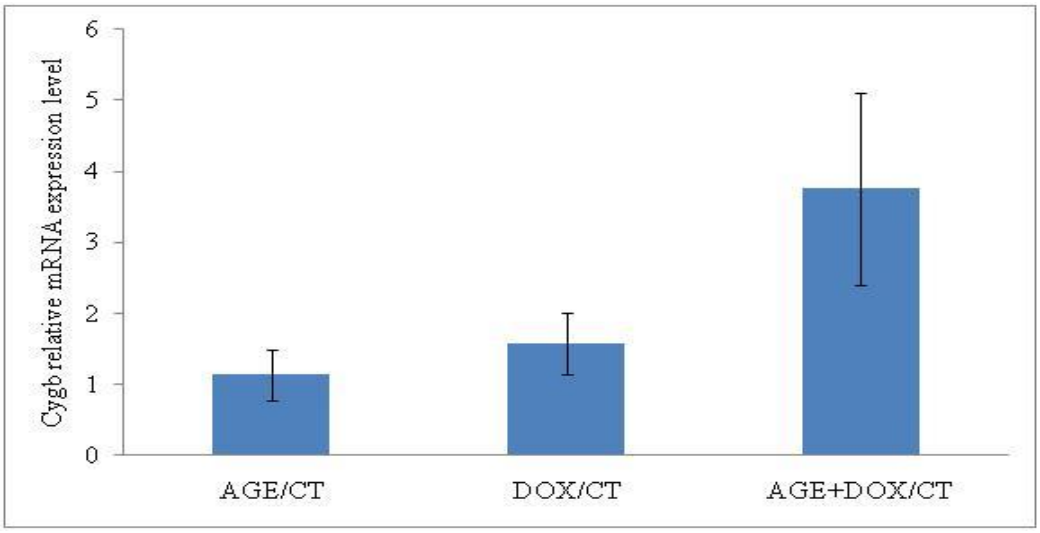
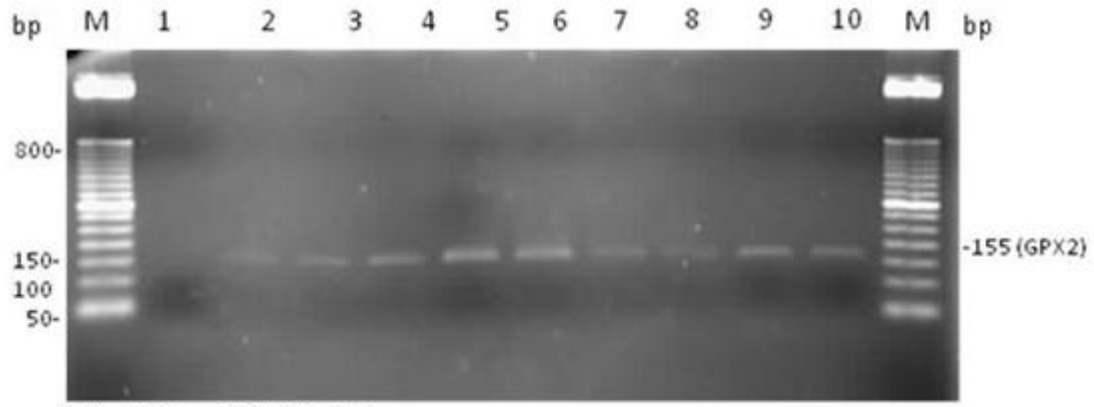


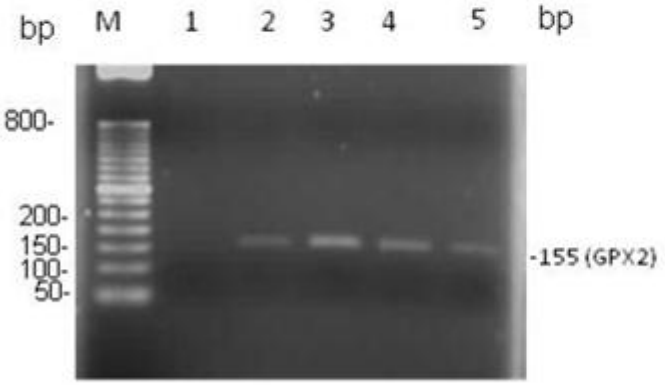
Figure 5.16: The mRNA expression levels of Cygb gene quantified by real-time qPCR. Each column represents the mean ± SEM (N=3). No significant differences between groups (p>0.05, one way ANOVA).

(A)



M: 50 bp DNA ladder
Lane 1: Blank (negative control)
Lane 2-4: Control
Lanes 5-7: DOX
Lanes 8-10: AGE

(B)



M: 50 bp DNA ladder
Lane 1: Blank (negative control)
Lane 2: Control
Lanes 3-5: AGE+DOX

Figure 5.17: Gel showing semi-quantitative RT- PCR results of Gpx2 gene expression in rat cardiac myocytes. PCR products were loaded on 2% agarose gel and stained with ethidium bromide. 50bp DNA ladder was used as marker.

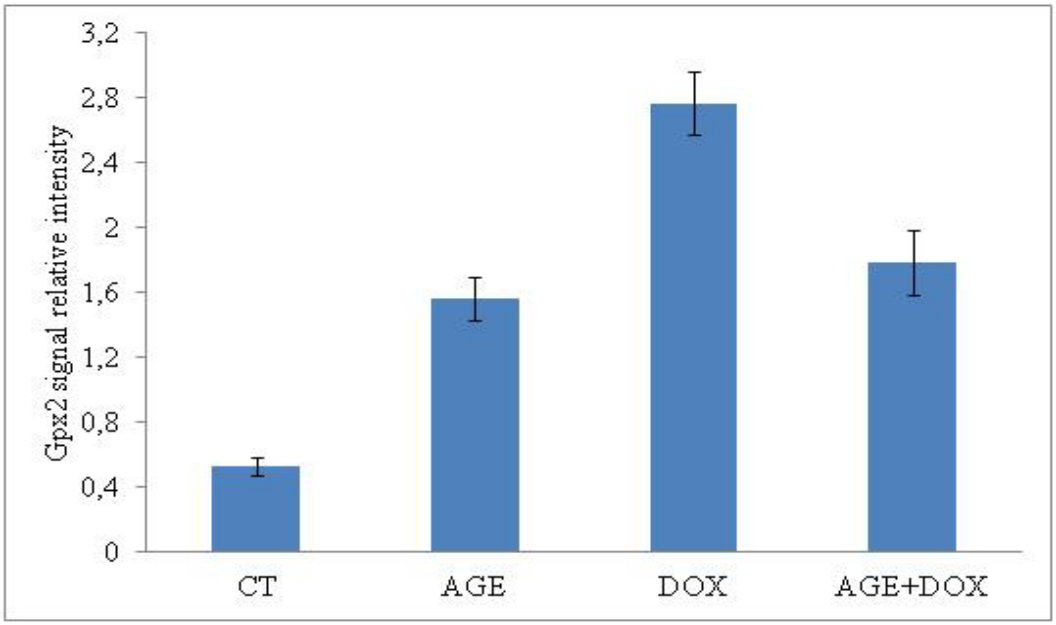


Figure 5.18:Semi-quantitative RT- PCR analysis of Gpx2 expression in rat cardiac myocytes. Results represent mean ± SEM (n=3). No significant differences between groups ($p>0.05$, one way ANOVA).

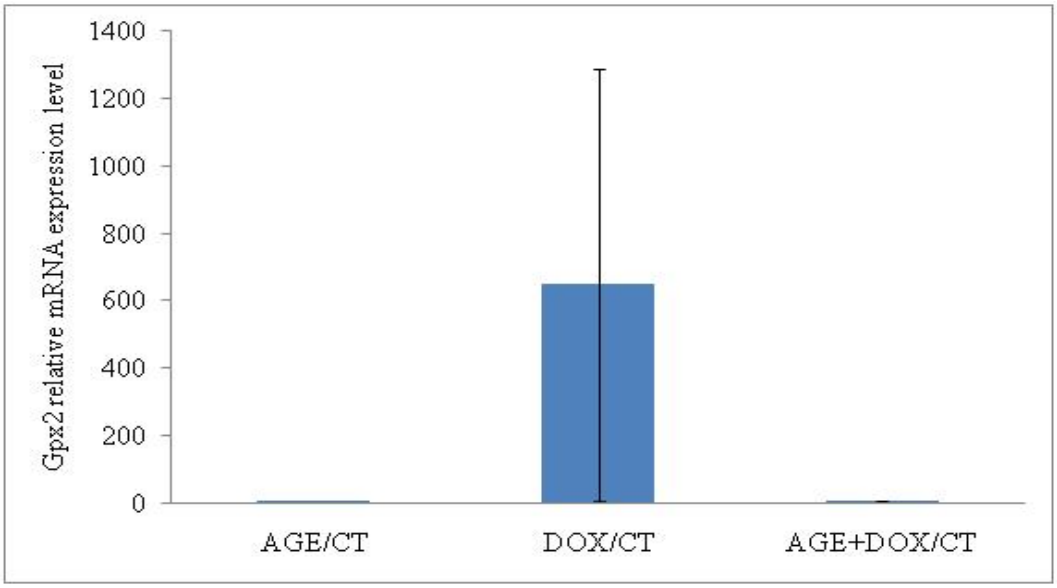


Figure 5.19: The mRNA expression levels of Gpx2 gene quantified by real-time qPCR. Each column represents the mean ± SEM (N=3). No significant differences between groups ($p>0.05$, one way ANOVA).

5.4 Discussion

The primary microarray analysis demonstrated that Ptgs2 is down-regulated in in DOX-treated cells. In this study, both semi-quantitative RT- PCR and real-time qPCR showed that expression of Ptgs2 was predominantly down-regulated in DOX-treated cells and to lesser extent in AGE+DOX-treated cells.

Researchers have found that prostanoids feedback regulate Cox-2 gene expression. However, prostanoid feedback regulation of Cox-2 gene expression depends on cell type and prostanoid product (Faour *et al*., 2001, Hinz *et al*., 2000). Prostaglandin E2 (PGE2) down-regulates Cox-2 expression in human umbilal vein endothelial cell (Akarasereenont *et al*., 1999)

Studies have identified Cox-2 in human cardiomyocytes in areas of myocardial infarction and in individuals with dilated cardiomyopathy, whereas no Cox-2 was found in normal hearts (Wong *et al*., 1998). Doxorubicin and H_2O_2 are reported to induce the expression of Cox-2 gene (Adderley and Fitzgerald, 1999).

Prostaglandins have been shown to have a cytoprotective role in several tissues, including stomach and heart (Balint, 1994, Kostic *et al*., 1997). The expression of Cox-2 protects against apoptosis and nitric oxide-mediated apoptosis (von Knethen and Brune, 1997, Morecki *et al.*, 1998). Song *et al.* (1996) reported that rats injected with lipopolysaccharide (a known inducer of Cox-2) are protected from ischemia/reperfusion injury.

Increased expression of Cox-2 gene has been found in failing human hearts (Wong *et al.*, 1998). Several studies have demonstrated that Cox-2 serves a protective function against

cardiac injury (Shinmura *et al*., 2000). There is elevated expression of Cox-2 in a variety of cancers (Kashfi and Rigas, 2005, Ranger *et al*., 2004)

The microarray analysis showed down-regulation of Gpx7 in DOX-treated cells. Pre-incubation of cells with AGE normalized Gpx7 gene expression. In contrast to microarray, semi-quantitative RT- PCR and real-time qPCR both showed Gpx7 expression was higher in DOX-treated cells compared to AGE+DOX treated cells.

Glutathione peroxidase (Gpx) is the antioxidant enzyme that scavenges hydrogen peroxide or organic hydroperoxides and thus protects cellular components against oxidative stress (Brigelius-Flohé, 1999). Glutathione peroxidase catalyses the reduction of hydrogen peroxide to water, with the simultaneous conversion of reduced glutathione to oxidised glutathione (Michiels *et al*., 1994).

Several studies have reported that DOX inhibits the activities of antioxidant systems, such as SOD and Gpx and decreases GSH content in myocardial tissue (Revis and Marusic, 1978, Julicher *et al*., 1986, Sazuka *et al*., 1989). Yin *et al*. (1998) investigated the effect of DOX on the activity and mRNA abundance of Gpx in mouse heart. A single i.p. injection of 15 mg/kg body of DOX was used. Four days after the treatment, DOX increased the levels of mRNA for Gpx in mouse heart while the activity of Gpx was not altered in the DOX-treated heart.

The primary microarray analysis showed down-regulation of Prdx5 in DOX-treated cells and to lesser extent in AGE+DOX-treated cells. Semi-quantitative RT-PCR demonstrated that Prdx5 expression was higher in DOX-treated cells compared to AGE+DOX-treated cells, while in real-time qPCR, DOX-treated cells showed a lower expression level than AGE+DOX-treated cells.

Xi *et al*. (2011) study found that upregulation of peroxiredoxin 5 by nitrate explained the reported enhancement of cardiac antioxidant defense by nitrate supplementation. Prdx5 may play a protective role against oxidative stress during this pathophysiological process

The microarray study demonstrated that Ucp3 is up-regulated in DOX-treated cells. Pre-incubation of cells with AGE normalized Ucp3 gene expression. In semi-quantitative RT-PCR no expression of UCP3 was detected.

It has been reported that UCP2 and UCP3 uncoupling proteins are involved in the age-depended heart dysfunction and development of the pathological mechanisms during ischemia-reperfusion (Hoshovska Iu *et al*., 2009). The mRNA expression of Ucp3 has been reported in many tissues (Boss *et al*., 1997, Vidal-Puig *et al*., 1997). It is commonly found in skeletal muscle and brown adipose tissue. Known factors that up-regulate Ucp3 mRNA expression are the thyroid hormone T3, cold exposure, fasting , non-esterified fatty acids, and hypoxia (Flandin *et al*., 2005, Larkin *et al*., 1997, Jekabsons *et al*., 1999, Gong *et al*., 2000)

The microarray analysis showed that Cygb is up-regulated in DOX and AGE+DOX-treated cells. Semi-quantitative RT- PCR demonstrated that DOX-treated cells showed higher Cybg expression than AGE+DOX-treated cells whilst real-time qPCR displayed higher Cygb expression in AGE+DOX- treated cells.

Nishi *et al*. (2011) found that Cygb function as a defensive mechanism against oxidative stress both *in vitro* and *in vivo*. Their study demonstrated that kidney ischemia-reperfusion (I/R) increased the number of Cygb-positive cells per area and up-regulated Cygb mRNA and protein expression in kidney cortex tissues. Likewise, hypoxia up-regulated Cygb expression in cultured rat kidney fibroblasts. A study by Hodges *et al*. (2008) found that

cytoglobin offers cytoprotection of neuronal cells from oxidative-related damage, for example, during ischaemic reperfusion injury following hypoxia. It has been shown that Cygb protects SH-SY5Y neuroblastoma cells from H_2O_2-induced cell death (Fordel *et al*., 2006)

Glutathione peroxidase 2 is up-regulated in both AGE+DOX- treated cells and DOX-treated cells. Both the semi-quantitative RT- PCR and real-time qPCR demonstrated expression of Gpx2 to be higher in DOX-treated cells compared to AG+DOX-treated cells.

A reduction in the activity of GPx enzyme is associated with the accumulation of highly reactive free radicals (Sheela and Augusti, 1995). Several studies have reported that DOX-induced cardiotoxicity can be inhibited by the overexpression of antioxidant enzymes such as MnSOD and catalase (Kang *et al*., 1996, Yen *et al.*, 1996). It has been demonstrated that an early and persistent decrease in Gpx1 after DOX treatment may contribute to DOX-induced cardiotoxicity (Li and Singal, 2000, Sazuka *et al*., 1989). It has been reported that the up-regulation of glutathione system is effective in the protection against oxidative cell injury (Zhu *et al*., 2007). It has been demonstrated that up-regulation of Gpx2 is a defence mechanism against severe oxidative stress (te Velde *et al*., 2008).

The results of this study demonstrated that using semiquntitative RT-PCR, AGE pre-treatment reduced insignificantly the increased gene expression of Gpx7, Prdx5 ,Cygb, and Gpx2 observed with DOX.

In conclusion, the results of this study have shown that DOX incubation with cardiac myocytes caused an increased expression of oxidative stress responsive genes. However, AGE pre-incubation seems to normalize the expression of some oxidative stress responsive genes. This suggests that AGE is useful for the prevention of DOX-induced cardiotoxicity.

Chapter 6 General Discussion

Doxorubicin is an anthracycline quinone that is widely used as a chemotherapeutic agent for treatment of several types of cancer, including breast cancer and leukaemia (Livi *et al*., 2011, Lipshultz *et al*., 2011). However, the administration of DOX is known to induce numerous cardiotoxic effects, including transient arrhythmias, nonspecific electrocardiographic abnormalities, pericarditis, and acute heart failure and somewhat limits its use (Singal *et al*., 2000, Keizer *et al*., 1990, Billingham *et al*., 1978, Bristow *et al*., 1978). The heart is thought to be more sensitive to DOX toxicity as a result of the large number of mitochondria, an increased amount of NADH dehydrogenase associated with complex I in these mitochondria, the affinity of DOX for the inner mitochondrial membrane phospholipid cardiolipin, and a lower peroxide detoxification capacity than that of the liver (Doroshow *et al*., 1980)

The mechanism by which DOX causes myocardial injury is not fully understood. Nonetheless, the free radical hypothesis of DOX toxicity has been steadily gaining support over the years. Acute or chronic DOX cardiotoxicity is reduced in transgenic mice overexpressing mitochondrial manganese superoxide dismutase (MnSOD), catalase, or cysteine-rich metallothioneins respectively (Yen *et al*., 1996, Kang *et al*., 2001, Sun *et al*., 2001), supporting the idea that oxidative stress mediates DOX cardiotoxicity.

The results of this study have shown that DOX induced marked acute cardiotoxicity 48 hours after DOX injection in rats. Doxorubicin-induced cardiotoxicity was manifested by increased serum cardiac enzyme levels. The results reported in this study were in agreement with previous studies (Ibrahim *et al*., 2010, Venkatesan, 1998, Tatlidede *et al*., 2009). Pre-treatment with AGE effectively prevented DOX-induced cardiotoxicity. A study by

Demirkaya *et al.* (2009) demonstrated the ability of AGE to protect against DOX-induced cardiotoxicity in rats.

In this study, the oxidative stress was obvious by the reduction in serum TAS and increased MDA production in the plasma and heart of DOX-treated rats. These finding were consistent with those of others (Antonio *et al*., 2005, Machado *et al*., 2010, Bulent *et al*., 2008). Moreover, AGE efficiently reduced the increase in MDA production in the plasma and hearts of rats treated with AGE + DOX. Furthermore, DOX-induced oxidative stress in rat cardiac myocytes measured as 8-isoprostane was reduced with AGE pre-incubation.

The protective effect of AGE against oxidative stress is due to its antioxidant potential. Several studies have documented the strong antioxidant activity of AGE (Drobiova *et al*., 2009, Aguilera *et al*., 2010, Al-Numair, 2009, Avci *et al*., 2008, Awazu and Horie, 2008). In the present study, the inhibitory effect of AGE on MDA production and the notciable increase in the TAS may be due to SAC. Kim *et al.* (2001) measured the antioxidant activity of AGE and SAC in hydroxyl radical and superoxide generating systems. They found that the formation of 5,5-dimethyl-1-pyrrolline N-oxide (DMPO) adduct of the hydroxyl radical was strongly inhibited by garlic extract and SAC in the H_2O_2 plus iron system which generates the hydroxyl radical. In addition, accumulation of superoxide generated in the xanthine oxidase (XO)/acetaldehyde system was reduced with AGE and SAC.

Pre-treatment with AGE did not interfere with the cytotoxic activity of DOX but increased its activity against tumour cells in mice bearing EAC. A possible explanation for AGE enhancement of DOX-cytotoxic activity may involve the inhibition of carcinogen binding to mammary cell DNA, augmentation of detoxifying enzymes and hindering of metabolic activating enzymes (Sumiyoshi and Wargovich, 1990, Amagase and Milner, 1993). It has

been reported that AGE has antitumour activity (Chang *et al*., 2005, Karasaki *et al*., 2001, Kyo *et al*., 1998, Lamm and Riggs, 2001, Malki *et al*., 2009, Milner, 1996, Sakamoto *et al*., 1997, Seki *et al*., 2000).

Several hypotheses have been proposed for the mechanisms of cardiotoxicity associated with DOX therapy. The most common theories proposed to explain DOX cardiotoxicity are free radical generation resulting in mitochondrial dysfunction and disruption of calcium homeostasis (Wallace, 2003, Berthiaume and Wallace, 2007, Hamza *et al.*, 2008). However, many studies have shown DNA damage as an early event in the toxicity induced by DOX in cardiac cells (Chua *et al.*, 2006; L'Ecuyer *et al.*, 2006, Nithipongvanitch *et al.*, 2007a; Nithipongvanitch *et al.*, 2007b).

It has also been suggested that a tumor suppressor protein p53 is a critical mediator of DOX cardiotoxicity. This notion is supported by the observation that DOX induces p53 accumulation in the heart and that either pharmacological or genetic ablation of p53 results in the attenuation of cardiotoxicity following DOX treatment (Liu *et al*., 2004, Shizukuda *et al*., 2005).

The results of this study have revealed that AGE protected rat cardiac myocyte against DOX-induced cardiotoxicity manifested as increased activity of p53, caspase-3 and cell death. It seems that AGE exerts its cardioprotective effect through both its antioxidant activity and inhibition of mitogen- activated protein kinase (MAPK)/ extracellular signal-regulated kinases (ERK) signalling pathway. Aged garlic extract has a strong antioxidant activity and its major constituent SAC is a potent free radical scavenger and inhibitor of ERK (Kim *et al.*, 2006). Padmanabhan and Stanely Mainzen Prince, (2007) have reported that oral pre-treatment of Wistar rats with SAC (100 mg and 150 mg/kg) was protective

against isoproterenol-induced myocardial infarction. They found that SAC reduced lipid peroxide products and improved the antioxidant status.

The present study has demonstrated altered antioxidant gene expression in DOX-treated cells. There was a trend of up-regulation of five antioxidant genes in DOX-treated cells. Pre-incubation with AGE seems to normalize the altered gene expression although not reaching statistical significance.

In conclusion, the present study is the first to show the protective effect of AGE against DOX-induced cardiotoxicity at both the *in-vivo* and *in-vitro* levels. Pre-treatment of rats with AGE minimised DOX- induced oxidative stress and preserved the morphological integrity of the heart. Moreover, AGE did not interfere with the antitumor effects of DOX. It enhances the cytotoxicity of DOX in tumour cells and guards the organs against DOX-toxic effects. Similarly, rat cardiac myocytes were protected from the oxidative stress and apoptotic damage with AGE pre-incubation.

Aged garlic extract reduced the activity of p53, caspase-3 and cell death in DOX-treated cells possibly through its inhibitory effects on MAPK/ERK signalling pathway. Furthermore, AGE has established strong antioxidant activity. The current findings contribute to the insights of DOX-induced cardiotoxicity and identify AGE as a potential therapeutic candidate.

Future work

Aged garlic extract appear to possess a number of biological effects in addition to its antioxidant activity. It may act by inhibiting MAPK/ERK signalling pathway. Further research is needed to elucidate the molecular mechanisms involved. Clinical trials on cancer patients receiving DOX treatment will be useful in confirming the protective role of AGE in human subjects.

Aged garlic extract may be considered as a potentially useful candidate in the combination chemotherapy with DOX to limit cardiotoxicity and augment DOX antitumor activity. Future studies should also consider performing Western Blotting for detection of protein expression of the altered genes with DOX treatment

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Appendix 1

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Research Ethics Committee

Approval Form

This is to certify that the research proposal entitled:

Therapeutic Potential of aged extract in protection against Doxorubicin induced cardio-toxicity

Submitted by:

Huda Mohammed Naher Al Kreathy (Postgraduate Student)
Collage of Medicine, KAU, has been reviewed by the committee with respect to protecting the rights and welfare of human subjects involved in the research project and/or experimental animals utilized. The methods employed are adequate for obtaining the information required and satisfy the required ethical principles and does not involve undue risk in the light of the potential medical benefits to be derived there from.

Decision:

The committee approves the above mentioned proposal as fulfilling the ethical requirements.

Professor Hassan A Nasrat

Chairman of the Research Ethics Committee

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Appendix 2

Food and Chemical Toxicology 48 (2010) 951–956

Contents lists available at ScienceDirect

Food and Chemical Toxicology

journal homepage: www.elsevier.com/locate/foodchemtox

Aged garlic extract protects against doxorubicin-induced cardiotoxicity in rats

Huda Alkreathy [a], Zoheir A. Damanhouri [a], Nessar Ahmed [c], Mark Slevin [c], Soad S. Ali [b], Abdel-Moneim M. Osman [a,*]

[a] Department of Pharmacology, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia
[b] Department of Anatomy, Faculty of Medicine, King Abdulaziz University, Jeddah, Saudi Arabia
[c] School of Biology, Chemistry and Health Science, Manchester Metropolitan University, MI5GD, Manchester, United Kingdom

ARTICLE INFO

Article history:
Received 22 October 2009
Accepted 5 January 2010

Keywords:
Doxorubicin
Aged garlic extract
Cardiotoxicity

ABSTRACT

Clinical uses of doxorubicin (DOX), a highly active anticancer agent, are limited by its severe cardiotoxic side effects associated with increased oxidative stress and apoptosis. In this study we investigated whether aged garlic has protective effects against doxorubicin-induced free radical production and cardiotoxicity in male rats. A single dose of doxorubicin (25 mg/kg) caused increased both serum cardiac enzymes LDH and CPK activities and a significant increase malonyldialdehyde (MDA) in plasma. However, pretreatment of rats with aged garlic extract (250 mg/kg) for 27 days before doxorubicin therapy, reduced the activity of both enzymes, and significantly decreased of MDA production in plasma.

Total antioxidant activity was increased after aged garlic extract administration. Histopathological examination of heart tissue showed that DOX treatment resulted in alteration of cardiac tissue structure in the form of peri arterial fibrosis and apoptotic changes in cardiomyocytes. Pretreatment with aged garlic extract for 27 days ameliorated the effect of DOX administration on cardiac tissue; cardiomyocytes looked more or less similar to those of control. However, still vascular dilatation, mild congestion and interstitial edemas were observed. Our results suggest that aged garlic extract is potentially protective against doxorubicin-induced cardiotoxicity.

1. Introduction

Doxorubicin (DOX) is an effective anthracycline antibiotic used to treat many human neoplasmas, including acute leukemias, malignant lymphomas, and a variety of solid tumors. However, its clinical uses are limited by dose-dependent side effect of cardiotoxicity, which may lead to irreversible cardiomyopathy and eventually heart failure (Shan et al., 1996). The cardiac toxic effects of DOX may occur immediately after a single dose, or several weeks to months after repetitive DOX administration. Several explanations account for the doxorubicin cardiotoxicity, e.g., free radical production, calcium overloading, mitochondrial dysfunction and peroxynitrite formation have been proposed (Olson and Mushlin, 1990; De Beer et al., 2001; Shuai et al., 2007). The semiquinone form of DOX is a toxic, short-lived metabolite and interacts with molecular oxygen and initiates a cascade of reaction, producing reactive oxygen species (ROS) (Davies and Doroshow, 1986). The free radical hypothesis is the most popular and is well documented. The precise mechanism of doxorubicin cardiotoxicity and related preventive approaches are under intensive investigations. For example if DOX cardiotoxicity is related to free radicals formation, compounds with antioxidant activity may protect against DOX-induced toxicities in hearts (Siveski-Iliskovic et al., 1995).

Garlic (*Allium sativum*) is used as a vegetable spice and medicinal herb. Recent research on garlic has used it in the form of tablets, fresh, dried raw, boiled and cooked preparation (Gorinstein et al., 2006). Commercially available garlic preparations in the form of garlic oil, powder and pills are also widely used for therapeutic purposes. Garlic exhibits a wide range of properties including immunomodulatory, hepatoprotective, antimutagenic, anticarcinogenic, and antioxidant effects (Horie et al., 1989; Kailash, 1996; Rahman, 2001; Al-Numair, 2009). Aged garlic extract (AGE) is an odorless garlic preparation produced by prolonged extraction of fresh garlic at room temperature for up to 20 months (Amagase et al., 2001; Borek, 2001; Kasuga et al., 2001; Banerjee et al., 2003). It has been shown to be the most useful garlic product as antioxidant and effective in medicine compared with other garlic preparations (Borek, 2001; Kasuga et al., 2001; Banerjee et al., 2003). AGE has been reported to have powerful antioxidant and free radical scavenging properties (Imai et al., 1994; Amagase et al., 2001; Borek, 2001). The protective effect of natural product containing aged garlic on DOX-induced cardiac injury is not clearly shown on the basis of antioxidant enzymes, however electron

* Corresponding author. Tel.: +966 26952000x20218.
E-mail address: moneimosman@hotmail.com (A.-M.M. Osman).

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doi:10.1016/j.fct.2010.01.005

microscopic study clearly demonstrated such positive effect (Demirkaya et al., 2009).

Therefore, this study is directed to investigate the protective effect of AGE against DOX-induced cardiotoxicity from biochemical and histopathological point of view.

2. Materials and methods

2.1. Reagents

Doxorubicin hydrochloride, was purchased from Sigma–Aldrich Co. (St. Louis, MO, USA).

AGE (Kyolic®) was kindly provided by Wakunaga of America (Mission Viejo, CA). It is prepared by soaking sliced raw garlic (*Allium satifum*) in 15–20% aqueous ethanol for at least 10 months at room temperature. The extract is then filtered and concentrated under reduced pressure at low temperature. The content of water-soluble compounds is relatively high while that of oil-soluble compounds is low. The AGE used in these experiments contained 28.6% extracted solids (286 mg/ml), and S-allyl cysteine, the most abundant water-soluble compound in AGE, was present at 1.47 mg/ml.

2.2. Animals

Wistar albino rats (8–10 weeks of age, 180–200 g body weight) were obtained from King Fahd Medical Research Center, King Abdul-Aziz University, Jeddah, Saudi Arabia. The animals were conditioned for 1 week at room temperature. A commercial balanced diet and tap water, ad libitum was provided throughout the experiment. This study was approved by the ethical committee of King Abdul-Aziz University Medical Faculty.

2.3. Experimental protocol

Twenty-four male Wistar rats were divided into four equal groups consisting of six animals each and housed in a room with regular light/dark cycle with free access to food and water.

Two groups (I and II), were used as a control and received normal saline, i.p. and distilled water p.o. (group I), and aged garlic extract 250 mg/kg orally (group II) for 28 days. Groups (III and IV) received a single i.p. dose of DOX (25 mg/kg) on day 27th, after successive administration of distilled water (0.5 ml, orally, group III) or aged garlic extract (250 mg/kg orally, group IV).

At the end of the experiment period (29 days), 48 h, after DOX injection, rats were anesthetized and blood samples were collected from ophthalmic artery in the orbital rim prior to sacrifice. Serum was separated and heart specimens were fixed in 10% formalin for histopathological examination.

2.4. Assessment of cardiac enzymes

Plasma total Lactate dehydrogenase (LDH) and total creatine phosphokinase (CPK) activities were determined using commercial kits from RANDOX, UK and SPINREACT, Spain, respectively. Total LDH activity was assessed according to the method of Henery (1974).

2.5. Determination of lipid peroxides (measured as MDA)

Frozen samples of heart were thawed, rinsed successively with 0.9% NaCl and with cold (4 °C) 20 mM Tris–HCl, followed by homogenization in Barnson Sonifier (250 VWR scientific, Danbury, CT, USA). The homogenates were diluted with cold 20 mM Tris–HCl and centrifuged (10 min at 4 °C, 3000 g). Spectrophotometric assays of malonyldialdehyde (MDA) were performed with an aid of Lipid Peroxidation Assay Kit (Calbiochem), in accordance with the instructions of the manufacturer.

2.6. Determination of total antioxidant status in serum

Total antioxidant status was determined using a quenching method ABTS (2,2-azino-di[3-ethyl benzthiazolin sulphonate]) radical cation (ABRS+) by antioxidants using a total antioxidant assay kit (Miller et al., 1993), obtainable from Randox NX2332 kit, Randox Laboratories, Crumlin, UK.

2.7. Histopathological examination of heart section

Formalin fixed heart embedded in paraffin wax was serially sectioned (3–5 μm section), and stained with hematoxylin and eosin, for only assessment of histopathological changes (Monnet and Christopher, 1999).

2.8. Statistical analysis

Results are expressed as the mean ± SEM. Comparison between different groups was carried out by one-way analysis of variance test (ANOVA) followed by LSD test. The statistical significance was accepted at a level of $P < 0.05$.

3. Results

3.1. Effect of AGE on DOX-induced cardiotoxicity

Treatments of rats with a single dose of DOX (25 mg/kg) resulted in a 185% increases in plasma CPK activities ($P < 0.001$). However, LDH activity showed a 48.5% increase compare to control (Figs. 1 and 2). Pretreatment of DOX-treated rats with AGE (250 mg/kg) for 27 days, resulted in a 73.7% decrease in plasma CPK activities ($P < 0.05$). Light microscopic examination of heart sections after 48 h of DOX treatment revealed fibrosis around arteries, apoptotic cells, loss of striation and an increase in inflammatory cells (Fig. 4). However, in the case of treatment with AGE for 27 days, DOX treatment resulted in mild interstitial edema, mild vascular congestion and normal appearance of nuclei and striation (Figs. 3 and 5).

3.2. Effect of AGE on DOX-induced changes on MDA levels in serum and heart homogenate

Figs. 6 and 7 show the effects of AGE treatment for 27 days before DOX on the levels of MDA in both serum and heart homogenate. A single dose of DOX-induced a 55.3% increase in MDA in heart homogenates while there was a only 27.1% increase in the case of AGE pretreatment.

There was a 12-fold increase in MDA in plasma after DOX treatment ($P < 0.001$) compared with only a 5-fold increase when AGE was given before DOX ($P < 0.05$).

3.3. Effect of AGE on DOX-induced changes in total antioxidant levels in serum

A single dose of DOX decreased in total antioxidant state by about 4% while AGE administration showed a15% increase (Fig. 8). Pretreatment of AGE before DOX nearly normalized the antioxidant levels in plasma (4% compared to control).

4. Discussion

Anthracyclines are used to treat a variety of cancers but are widely associated with irreversible cardiomyopathy. The mechanism of doxorubicin-induced oxidative stress is the formation of an anthracycline-iron (Fe^{2+}) free radical complex. The latter reacts with hydrogen peroxide to produce hydroxyl ($OH^{\cdot}$) radical (Sugioka and Nakano, 1982). The iron chelators and free radical scavengers might provide cardiac protection by preventing the formation of the extremely reactive hydroxyl radical and by scavenging radicals that have been formed. The iron chelator ICRF-187 has been shown to protect against DOX-induced cardiotoxicity during experiments. However, its clinical success is limited because it increases the hematotoxicity in cancer patients (Sparano, 1998; Speyer et al., 1992). This study was directed to investigate the role of AGE which has been used as both food and medicine in many cultures for thousands of years (Ackermann et al., 2001; Dillon et al., 2003) for its antioxidant properties for cardioprotection against DOX-induced cardiotoxicity.

A single dose (25 mg/kg) of DOX-induced marked acute cardiotoxicity in rats 48 h after treatment. This was demonstrated by increase plasma CPK and LDH activities and confirmed by moderate histopathological changes in the heart including periarterial fibro-

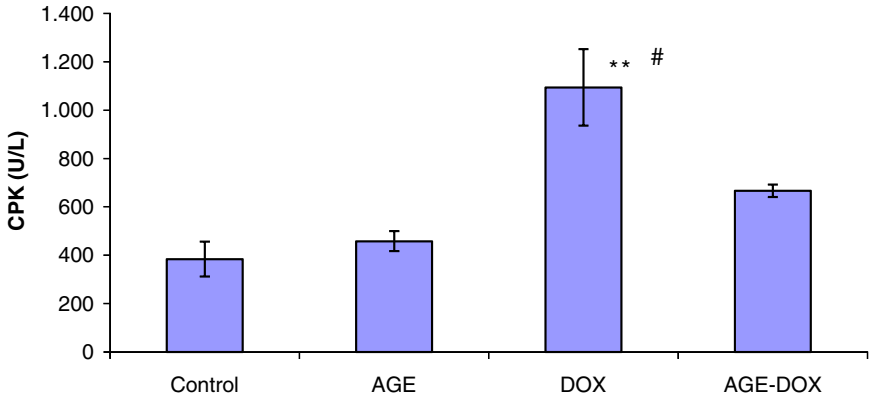

Fig. 1. Effect of DOX (25 mg/kg) alone or after pretreatment with AGE on the activity of cardiac enzyme CPK. Data are expressed as mean ± SEM ($n = 6$). **Significantly different from control ($P < 0.001$). #Significantly different from AGE + DOX ($P < 0.05$).

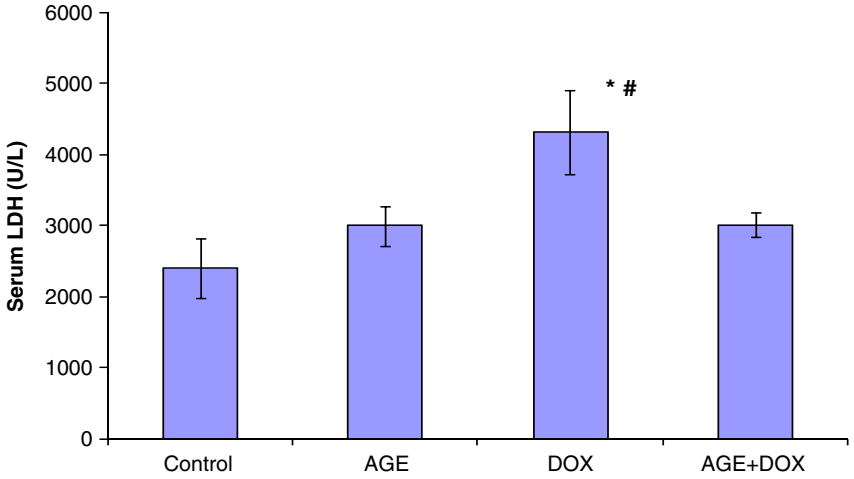

Fig. 2. Effect of DOX (single dose 25 mg/kg, i.p.) alone and after pretreatment with AGE (250 mg/kg, p.o.) on serum LDH activity (U/L) of male Wistar rats. The values expressed mean ± SEM ($n = 6$). *Significantly different from control ($P < 0.05$). #Significantly different from AGE + DOX ($P < 0.0$ 5).

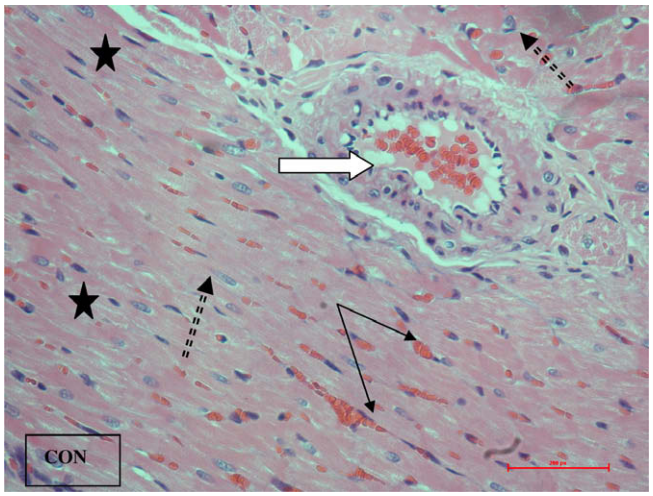

Fig. 3. Histological section from the left ventricle showing normal cardiomyocytes (black stars), with oval vesicular central nuclei, thin wall blood capillaries, and a branch of coronary artery were seen among the cardiac fibers (H&E ×40).

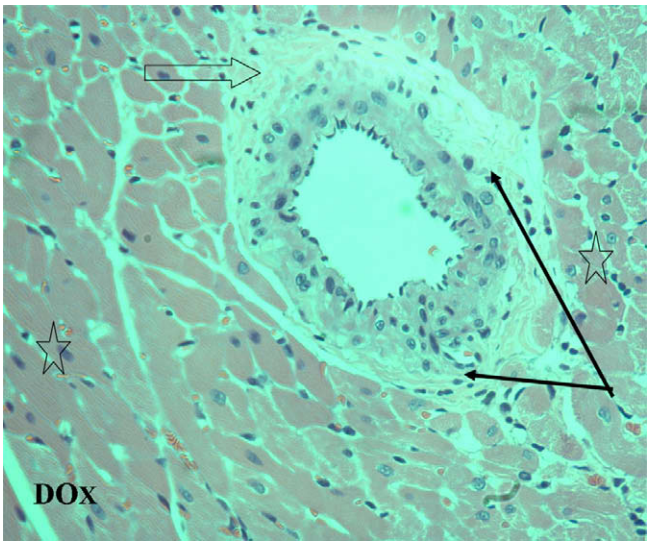

Fig. 4. Light micrograph of a part of rat heart treated with Doxorubicin (DOX) showing peri arterial fibrosis, loss of striation and increase in inflammatory cells. (H&E 40×).

sis, apoptotic cells, Loss of striation and increase in inflammatory cells. It is well known that the magnitude of CK and LDH activities in blood after myocardial injury reflects the extent of damage in its musculature (Preus et al., 1988). The mechanism of DOX-induced cardiotoxicity has been reported to be through formation of superoxide anions and their derivatives, particularly highly reactive and damaging hydroxyl radicals, which induces peroxidation of cell

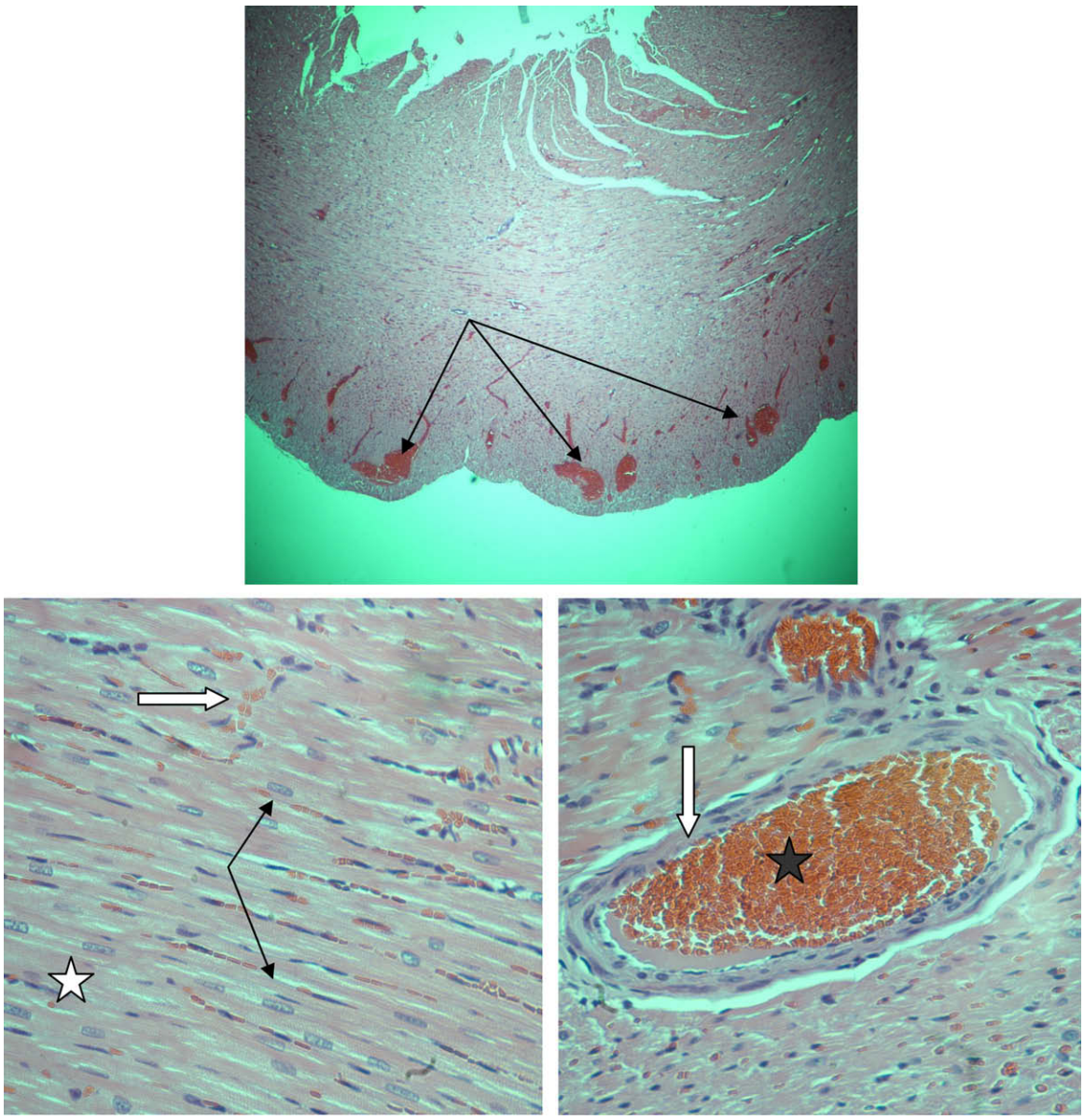

Fig. 5. Photomicrographs from AGE + DOX treated cardiac tissue. Notice the increase in sub-pericardial vascularity (arrows) in the upper figure. The lower left figure show normal cardiomyocytes with normal appearance of nuclei and striation (star). The nuclei of the cells are oval vesicular and central. Normal but dilated congested vessels among cardiac fibers were seen (right figure) (H&E 40×).

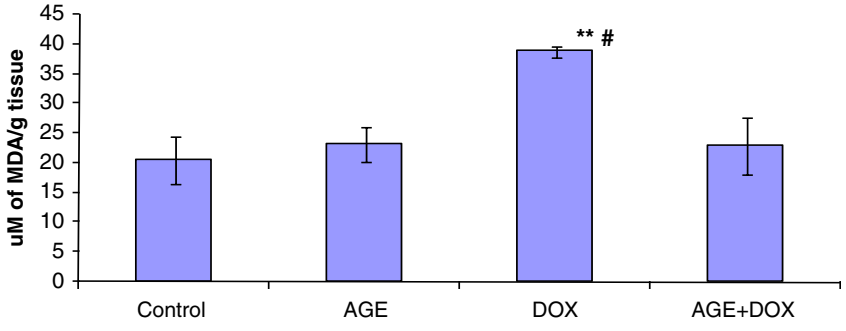

Fig. 6. Effect of DOX (single dose 25 mg/kg, i.p.) alone and after pretreatment with AGE (250 mg/kg, p.o.) on heart homogenate malonyldialdehyde (MDA) activity (μM/g tissue) of male Wistar rats. The values are represented by mean ± SEM ($n = 6$). **Significantly different from control ($P < 0.001$). $^{\#}$Significantly different from AGE + DOX ($P < 0.05$).

membrane lipid (Hemnani and Parihar, 1998). Our results are in agreement with others (Van Vleet et al., 1980; Tesoriere et al., 1994; Nagi and Mansour, 2000; Al-Majed et al., 2002; Yagmurca et al., 2003), who all reported cardiac toxicity after DOX treatment.

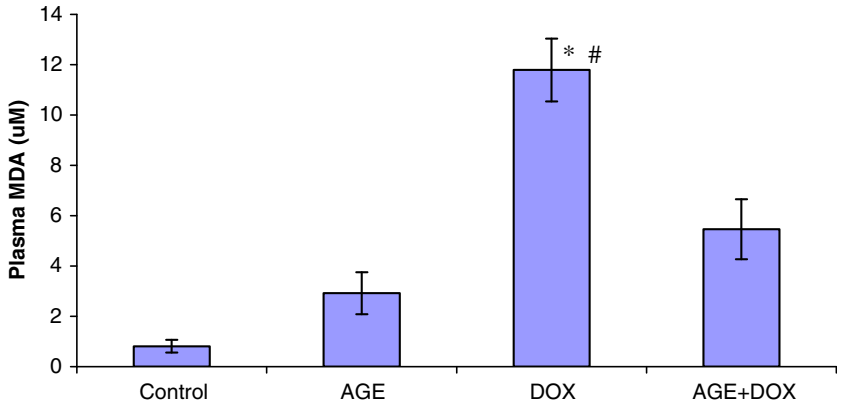

Fig. 7. Effect of DOX (single dose 25 mg/kg, i.p.) alone and after pretreatment with AGE (250 mg/kg, p.o.) on plasma malonyldialdehyde (MDA) activity (μM/g tissue) of male Wistar rats. Data are expressed as means ± SEM ($n = 6$). *Significantly different from control ($P < 0.05$). #Significantly different from AGE + DOX ($P < 0.05$).

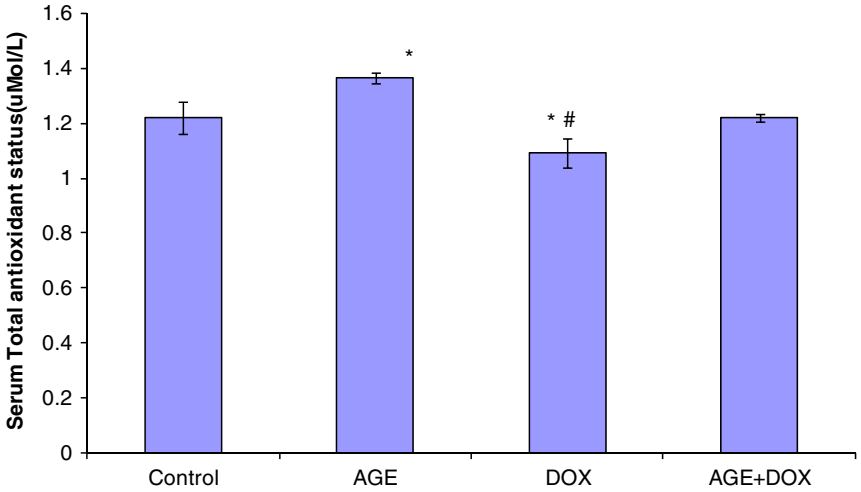

Fig. 8. Effect of DOX (single dose 25 mg/kg, i.p.) and/or AGE pretreatment (250 mg/kg, p.o.) on serum total antioxidant status (TSA) activity (μmol/l) of male Wister rats. The values are represented by mean ± SEM ($n = 6$). **Significantly different from control ($P < 0.001$). #Significantly different from AGE + DOX ($P < 0.05$).

Other investigators have observed generalized tissue damage to other organs following DOX treatment (Yoda, 1986; Bagchi et al., 1995). The mechanism of DOX-induced cardiotoxicity has been implicated by many investigators. In term of specific organ toxicity, lipid peroxidation and formation of peroxinitrite have been involved in the pathogenesis of DOX-induced cardiac toxicity (Myers et al., 1977; Singal and Pierce, 1986; Pacher et al., 2003). In our study a significant increase in lipid peroxidation in term of MDA in plasma (Fig. 7) has been observed after DOX treatment.

The increase in CK activity following acute DOX administration was significantly prevented by garlic treatment continuously for 27 days before DOX. It is well known that garlic is medicinally used since ancient times as antioxidant and it has been shown that chronic intake of garlic enhanced Superoxide dismutase and catalase activities in heart tissue which offers protection against oxidative stress associated with ischemic reperfusion injury (Banerjee et al., 2002a,b; Mukherjee et al., 2003).

At the same time Borek (2001) reported that storing sliced raw garlic produces AGE with increased activity of certain new compounds such as S-allylcysteine and S-allylmercaptocysteine, allixin and selenium which are stable, highly bioavailable and significantly antioxidants.

Moreover, garlic was reported to reduce chemotherapy side effects such as heart and intestinal damage commonly seen with certain anticancer agents (Al-Numair, 2009).

The biochemical data were confirmed by histopathological data which showed mild myocardial injury induced by DOX after pretreatment of rats with AGE (Fig. 5). It has been reported that cardiac histopathological changes were induced by DOX treatment which confirm our presented data (Van Vleet et al., 1980; Tesoriere et al., 1994; Monnet and Christopher, 1999). Moreover, Borek (2001), showed that AGE protects cardiac cells in vitro against DOX-induced cardiotoxicity, our results confirms this data where AGE pretreatment showed a decrease in DOX-induced elevation of MDA production in both plasma and heart tissue (Figs. 6 and 7).

In conclusion AGE contains a wide range of antioxidants that protect against cardiac damaging effects of DOX. Additional human studies using AGE and its constituents to further elucidate their protective role against agents that induce tissue damaging effects and necessary at the same time, the molecular studies to reveal the underlying mechanisms.

Conflict of Interest

The authors declare that there are no conflicts of interest.

Acknowledgments

This work is a part of thesis prepared at King Fahd Medical Research Center, Jeddah,Saudi Arabia.

Financial support was obtained from the Scientific Channel Program, King Fahd Medical Center, King Abdulaziz University.

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